

# PRACTICAL BIOLOGICAL CHEMISTRY

GUIDE POUR LES MANIPULATIONS DE  
CHIMIE BIOLOGIQUE

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## TRANSLATOR'S PREFACE

I FIRST became acquainted with this work of MM. Bertrand and Thomas when stationed at Genoa in the summer of 1918, and though myself requiring it for special purposes, was at once struck with the very wide field which it covers.

As the authors state in their original preface, the subject of Biological Chemistry is one which daily finds increasing spheres of application, and consequently an increasing number of students. For the specialist it of course has an especial fascination of its own, while to the physiologist and biologist it has long been indispensable. Recent experience has shown that many of the problems which come within the cognizance of the physician and the pathologist are no less dependent upon its principles and methods for their elucidation, while scientific agriculture and a host of commercial processes open up numberless fields of inquiry in the same direction.

The present work is also of outstanding value since the experiments described are of fundamental practical importance, as may be seen by a glance at the table of contents. To the physician, physiologist, and pathologist the descriptions of the analysis of the blood, urine, and the fatty matters present in animal tissues will be of interest. To the botanist and agriculturalist the analysis of vegetable ash and the various constituents of plant tissues will make a special appeal, while the sections on essential oils, tannins,

alkaloids, and the various fermentations and their products will interest those who are applying science to the furthering of commercial enterprise.

In brief, the cardinal feature of the work is its essentially broad and practical character, which cannot fail to be of service to those who contemplate a study of bio-chemistry for any of the purposes we have suggested. It is of course not intended as a laboratory handbook for the specialist in pathological bio-chemistry or any other particular branch of the subject, but is designed rather to afford a comprehensive scheme of elementary work over a wide field, so as to ensure a broad foundation for future investigations in whatever branches of the subject the student may afterwards concentrate upon.

A full and detailed description, together with the necessary tables, is given of Professor Bertrand's method of estimating sugars, while the section on enzymes proceeds from the pen of an acknowledged master of the subject.

My own part in preparing the translation has been a very small one and strictly limited to presenting the work of the French scientists to those who are unable to study their work in the original. Here and there an explanatory note has been added, and the whole work has been submitted to Professor Bertrand for his approval before publication. I can conscientiously say that I have endeavoured to the best of my ability to preserve the conciseness and lucidity of expression which mark the original work, and are of course characteristic of the works of all eminent French scientists.

In conclusion, it is my pleasant duty to express my very sincere thanks to those friends who have been kind enough to aid me with their assistance, and especially to Professor Sidney Russ of the Middlesex Hospital and to Dr. Arthur

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Compton of the Institut Pasteur, both of whom have read, through the whole of the proof-sheets, and to Professor Bedson of Armstrong College, Newcastle-on-Tyne, for much valuable advice in the earlier stages of the work. My acknowledgements would be incomplete without a cordial recognition of the assistance I have received from my publishers, for their uniform kindness during the unexpectedly protracted process of publication, and for the loyal spirit of co-operation in which they have carried out their share of the work.

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forming what is known as a "blank experiment," in which the same reagents (and in the same quantities) are used as in the actual analysis, only the substance under investigation is not added. If such a blank experiment reveals the presence of the substance we desire to investigate as an impurity in the reagents, clearly these must either be rejected or purified. Sometimes it is possible to perform the blank experiment quantitatively, and to make the necessary allowances for the impurities when calculating our final results.

Finally, we may briefly indicate the precautions which must be observed to ensure accuracy, and to avoid damage to apparatus and risk of injury either to the experimenter himself or to others.

1. Except where special instructions are given to the contrary, avoid using excess of reagents. A pipette or a drawn-out glass tube are useful for liquids, as they can then be added drop by drop if desired.

2. When taking readings with any form of graduated glass apparatus (burettes, graduated flasks, etc.), always take care that the line of vision just cuts the *bottom* of the meniscus at the top of the fluid.<sup>1</sup>

3. When measuring a liquid by means of a pipette, the narrow end should be placed against the side of the vessel into which the liquid is transferred when reading the level in the pipette and stopping the flow of liquid. By this means the liquid runs freely and the volume delivered corresponds to that indicated by the graduation of the pipette.

4. When making accurate weighings we may avoid errors due to the influence of the hygrometric condition of the atmosphere upon the vessel which contains the material

<sup>1</sup> When dealing with opaque liquids (*e.g.* permanganate solution, milk, etc.) the reading may be taken from the top of the meniscus, provided that the difference in level is allowed for by measurement with a burette or suitable pipette.

to be weighed, by using a second exactly similar vessel as a counterpoise.

5. Pay strict regard to the scrupulous cleanliness of all apparatus such as the microscope, spectroscope, and polarimeter. Should a drop of some reagent fall upon a balance-pan or microscope stage, wipe it off immediately, and always see that the microscopic objectives are not soiled by contact with any liquid used in mounting preparations, except, of course, in the case of special immersion objectives. If a lens is soiled and simple wiping is insufficient to clean it, do not use reagents for cleaning purposes at random, but *always ask what to use and how to use it*, otherwise the cement which is used to fix the lenses may be softened and the lens will fall out. Microscopic objectives may be wiped either with a perfectly clean thin piece of silk which is kept, away from dust, exclusively for this purpose, or with a piece of soft Japanese paper.

When using the balance, never under any circumstances put the material to be weighed directly upon the scale-pan. It is useful to keep a series of tared watch-glasses for this purpose, with the approximate weight of each marked upon it. Never touch the weights with the fingers, but always make use of the pair of forceps provided and kept only for the purpose. It is also very important that no changes should be made in the load of either scale-pan when the balance is swinging, but always when it is set at rest.

6. Graduated glass apparatus must also be kept scrupulously clean, and one should never *blow* down a pipette, which introduces fatty substances. Graduated glass vessels should be used exclusively for measuring purposes; they should not be heated more than necessary, and hot liquids should not be poured into them. The last observation applies particularly to all vessels made of thick glass, as they may thereby be broken.



## INTRODUCTION

WE think it may be useful to indicate in the first place certain principles which will enable beginners in the study of biological chemistry to use this book to the best advantage.

1. Before attempting to carry out any experiment in the laboratory, always make certain that you fully understand the theoretical principles upon which the practical work is based. Then carefully read through the description of the experiment so as to get a thorough grasp of all its details.

2. If the first attempt at carrying out any experiment fails, repeat it until success is attained. When you have thoroughly mastered the technique of any given experiment, it is a good plan to repeat it several times with smaller and smaller amounts of the reagents so as to obtain a practical idea of the limits of sensitiveness of the different reactions. If the subject of investigation is a quantitative estimation, it is a good plan to repeat it with the addition of such precautions as may ensure increased accuracy. Generally speaking, when a result is obtained which is within one per cent of that calculated it may be regarded as a good approximation; it should be noted, however, that in many cases a more accurate determination can—and should—be obtained.

3. With respect to this question of accuracy we must carefully bear in mind that there is a vast difference between absolute and relative error in the conclusions to be drawn from any given experiment. In some cases the absolute

error is of outstanding importance, while in others the relative error is the important feature. For instance, if we are investigating the amount of some substance which is present in an organic material only in very small quantities, it is clear that the absolute error (represented in this case by the limit of sensitiveness of the reaction) should be smaller than the amount of the substance under investigation, otherwise it could not be detected, and the conclusion would be negative instead of positive.

If, on the other hand, we are dealing with the estimation of the quantity of some easily titrated substance in a mixture, the absolute error may be of comparatively slight importance, since the amount of experimental material can always be so increased as to give a good approximation. In such cases it is the relative error which determines the limit of precision of the experiment.

4. It is impossible to urge too forcibly the advantages of performing a control experiment whenever this is practicable, using for this purpose some convenient amount of a substance of known composition which contains the element which we are investigating or estimating. For example, if we are estimating the amount of iodine present in thyroid-gland tissue, a control experiment should be devised, using a very dilute solution of potassium iodide. If the quantity of phosphoric acid in some animal or vegetable tissue forms the subject of our investigations, a control experiment may be performed upon some stable phosphate of known and definite composition, such as acid ammonium phosphate, which is stable and is, moreover, neither efflorescent nor hygroscopic.

5. Another precaution which is specially necessary in delicate experiments involving the estimation of very small quantities is to make certain that we are working with absolutely pure reagents. This may be done by per-

7. When a piece of apparatus is exhausted by the filter-pump, do not forget to disconnect it from the pump before turning off the water. If this is not done, water will be sucked back into the apparatus.

8. The distillation of many liquids is accompanied by "bumping," and portions of the contents of the distilling flask are spurted over into the condenser. Both these troubles can be overcome by putting some scraps of pumice (or, better, fragments of wood such as broken match-sticks) into the distilling flask.

If the process of boiling is accompanied by too copious frothing, the addition of a few drops of oil will cause the froth to disappear.

9. When a liquid is boiled in a test-tube, the tube should be gently shaken throughout the process, so as to avoid the contents of the tube being suddenly spurted out. As spurting will sometimes occur in spite of all precautions, it is obvious that the tube should *invariably* be held so that such accidental spurtings may do no damage either to persons or things.

10. When lighting the burners of an apparatus which is heated by gas (such as an autoclave, etc.) an explosion may be caused if there is much delay between turning on the gas-tap and applying the light.

The correct way to light burners of this kind is, *First*, to turn on the gas-tap for a few seconds so as to drive any air out of the burners themselves; *Secondly*, to turn off the gas; *Thirdly*, to apply the light to the burners and then immediately to turn the gas on again. This procedure will eliminate any chance of an explosion, and will also prevent the burners from "lighting back."

"Lighting back" can usually be noticed from the appearance of the flame and from the offensive smell which is produced. It is important to avoid this, not only because

the gases are poisonous and also the apparatus is rendered inefficient, but because in some cases (*e.g.* ordinary Bunsen burners) the lower part of the burner may become so hot that the rubber connecting pipe is melted and there is a very considerable danger of fire.

11. Never carry out any manipulation with explosive or inflammable liquids (such as absolute alcohol, ether, and especially carbon disulphide) near a naked light, and remember that the vapours of these liquids may be ignited by a lighted cigarette.

If a liquid of this kind has to be evaporated, every precaution must be taken, such, for example, as heating on a water-bath filled with hot water which is renewed from time to time as required, thus avoiding any risk of ignition from a naked flame in the neighbourhood.

12. Should any reagents or apparatus take fire, the fire can be extinguished in different ways, according to circumstances. If it occurs at the mouth of a vessel with a narrow neck, covering with a damp folded duster will be enough; if an evaporating basin or some other wide-mouthed vessel is the cause of the trouble, it may be covered with an earthen vessel or a bucket. If these measures are insufficient, *water* may be thrown on if the fire is due to alcohol, or *sand* in the case of ether, benzene, carbon disulphide, or other substances which are insoluble in water, and sand should, of course, also be used when metallic sodium or potassium is in question.

If burning liquid is spilt on the clothes, damp cloths form the most convenient method of extinguishing it.

13. When working with bodies which give off poisonous or irritating fumes, such as bromine, acetyl chloride, or benzoyl chloride, it is best to conduct operations either in the open air or in a fume chamber furnished with a good draught. It is well to have close at hand in the fume cup-

board some form of support or a test-glass in which the flask or test-tube can be placed upright if the fumes are overpowering.

14. When dealing with living cultures of micro-organisms take care that none is spilt on the bench, clothes, or hands. If such a "spill" or soiling by a culture should occur, an antiseptic solution should at once be applied (1 per cent formalin or a one-in-a-thousand solution of mercuric chloride); this precaution should always be taken even if the cultures are not regarded as pathogenic. Vessels which have contained cultures can be sterilized either by boiling with a dilute solution of alkali or by heating in the autoclave. Finally, remember that if one is smoking while dealing with cultures of micro-organisms, pipes and cigarettes must not be laid upon the bench nor touched with soiled fingers. A bowl of suitable antiseptic (1 in 1000 mercuric chloride) should always stand upon the bench when working with living cultures, and all cultures should be treated as if they were pathogenic.

## NOTE TO THE READER

1. Potash pastilles or pellets. These are little globules of potassium hydrate each weighing about .2 to .25 gramme, and are made by fusing ordinary caustic potash and allowing it to fall drop by drop on to a suitable plate. They are very handy in laboratory work and obviate the tiresome necessity for breaking off little pieces of stick potash. Needless to say, they must be preserved in a perfectly dry bottle fitted with a paraffined stopper or a cork soaked in paraffin wax.

2. Strength of acids and alkalies measured in Beaumé degrees, *e.g.* (36° B.). The tables of percentage composition of solutions and corresponding specific gravities will be found at pp. 322-328.

# FIRST PART

## STATICS

### CHAPTER I

#### DETECTION AND ESTIMATION OF ELEMENTS

##### Detection of Carbon

1. ORGANIC compounds are characterized by the presence of carbon in combination with hydrogen, and nearly always with other elements, such as oxygen and nitrogen. To distinguish them from "inorganic" or "mineral" bodies it is necessary to establish the presence of carbon.

Organic bodies are combustible; they burn when brought in contact with a flame in the presence of air. On the other hand, if strongly heated in a test-tube, they usually become "carbonized," assuming first a brown and then a black colour, due to the liberation of free carbon. This character is not invariable; for instance, oxalic acid,  $(\text{COOH})_2 + 2\text{H}_2\text{O}$ , is decomposed by heat without carbonization; while cupric hydrate,  $\text{Cu}(\text{OH})_2$ , blackens on heating, owing to the formation of anhydrous cupric oxide.

2. The presence of carbon is definitely shown by the evolution of carbon-dioxide, when the substance under examination is strongly heated with copper oxide, the latter undergoing reduction to metallic copper. The procedure is as follows.

Introduce a mixture of .1 to .2 gramme of the sub-

stance under examination, and 5 grammes of powdered copper oxide into a test-tube, which is then fitted with a cork and delivery tube, which is bent at a right angle. The free end of the delivery tube is kept beneath the surface of a solution of barium hydrate, and the contents of the test-tube cautiously heated to dull redness. In the presence of carbon in the material examined, the barium hydrate solution becomes turbid, owing to the formation of insoluble barium carbonate. The delivery tube must be withdrawn from the liquid before the test-tube cools, in order to avoid back suction, and consequent fracture of the tube.

It must be remembered that some carbonates disengage carbon-dioxide on heating; these, however, may be distinguished from organic bodies by their effervescence when treated with hydrochloric acid.

3. Organic bodies when heated with a mixture of chromic and sulphuric acids reduce the chromic acid; the original yellow colour of the mixture being changed to green, owing to the formation of green chromium sulphate.

Put a few centigrammes of the material in a test-tube; add 5 cc. of concentrated sulphuric acid, and 1 cc. of a 2 per cent solution of potassium bichromate; mix by gentle shaking. Should the original yellow colour persist, heat cautiously, but not to boiling. An olive or bright green coloration indicates the presence of carbon, provided, of course, that no inorganic reducing bodies are also present.

### Detection of Nitrogen

4. Generally speaking, but not invariably, organic nitrogenous bodies give off an odour of burnt hair when strongly heated. The presence of nitrogen is put beyond question by the formation either of ammonia or of a cyanide, and the subsequent detection of these bodies.

5. *Formation of Ammonia by heating with Soda-lime* (Will and Warrentropp).—Introduce a mixture of about 2 gramme of the experimental material and 3 to 4 grammes of soda-lime into a hard test-tube, carefully wiping the



mouth of the tube with a piece of filter-paper to remove any adherent particles of soda-lime. Heat to dull redness, and hold a moistened red litmus-paper to the mouth of the tube; in the presence of ammonia, the litmus-paper turns blue. Ammonia can also be detected by its odour, or by the formation of white fumes of ammonium chloride when a glass rod moistened with strong hydrochloric acid is presented to the mouth of the tube. For the reactions of ammonia see § 528.

6. *Detection of Nitrogen by the Formation of Sodium Cyanide* (Lassaigne).—Take a small test-tube (about 10 cm. by .5 cm.), and introduce a fragment of the substance to be tested (about the size of a pin's head). Then add a little piece of sodium, about four times this size, and heat until the metal fuses, which occurs about a dull red heat. Plunge the still hot tube into about 5 cc. of water in an evaporating basin; the tube breaks and any unaltered sodium takes fire. The contents of the tube are dissolved, and the liquid filtered, the resulting filtrate being tested for the presence of a cyanide.

The procedure needs care.<sup>1</sup> Throughout the whole process the mouth of the tube must be directed away from the face of the operator to avoid any risk from ejected fragments of sodium, and the heating must be continued long enough to destroy any tarry matters which would interfere with the production of a clear solution. In the presence of sodium, the carbon and nitrogen of the organic body produce sodium cyanide, the presence of which is shown by the following reactions.

7. Half the solution obtained as described above is gently warmed with a small crystal of ferrous sulphate (about the size of a pin's head), and the mixture gently shaken in the presence of air. The solution, which should

<sup>1</sup> If only the very small amounts of sodium and test-material mentioned are used, it is quite safe to break the hot tube in water as described. Should these amounts be exceeded, however, and especially if any free sodium is obviously present, it is safer to allow the tube to cool, add a few drops of absolute alcohol (which combines with any free sodium present), and then to add the water.—[TRANSLATOR.]

be alkaline, is cooled and then acidified with dilute hydrochloric acid, using a scrap of litmus-paper as indicator. The formation of a blue precipitate (or colour, if very dilute) indicates the presence of a cyanide. This blue precipitate or coloration is due to the formation of "Prussian blue," produced by the interaction of the ferrocyanide formed, with that part of the sulphate of iron which has been oxidized by shaking with air.

If the colour does not appear, a drop of *dilute* ferric chloride solution is added, in order to ensure the presence of sufficient iron. In doubtful cases the bluish liquid may be filtered on a small filter-paper, which retains any precipitate and assumes a blue tint.

8. Heat the other portion of the solution to boiling for a minute or two, with three or four drops of yellow ammonium sulphide. After cooling, acidify with dilute hydrochloric acid; boil to expel sulphuretted hydrogen, filter to remove the precipitated sulphur, and cool under the cold-water tap. Add, drop by drop, a dilute solution of ferric chloride; a red colour indicates the presence of a thiocyanate, and therefore of a cyanide in the solution tested.

The "sodium method" is more delicate than the "soda-lime method," except in the case of certain oxygen-containing alkaloids.<sup>1</sup>

In all these reactions it is necessary to avoid undue dilution, by only taking just as much of the various reagents as is necessary to attain the desired result.

### Detection of Sulphur

9. The presence of sulphur can be demonstrated by the formation either of a sulphide or of a sulphate.

*Formation of a Sulphide.*—Proceed as directed for the detection of nitrogen by the sodium method in § 6. To the filtered alkaline solution add a few drops of a dilute

<sup>1</sup> These methods for the detection of nitrogen are, of course, not suitable in the case of organic nitrates and nitro-compounds. These give off nitrous fumes on heating, and moreover frequently explode.

solution of sodium nitroprusside. A violet colour indicates the presence of sulphur.

10. *Formation of Lead Sulphide*.—This reaction is only available with certain compounds such as some proteins, cystine, etc. Introduce into a test-tube a few drops of lead acetate solution; then add sufficient of a 10 per cent solution of sodium hydrate to re-dissolve the precipitate of lead hydrate which is first formed. Add a small quantity of the material to be investigated, and boil gently for a few minutes. Blackening, due to the formation of lead sulphide, indicates the presence of sulphur. This reaction is less delicate than the foregoing (§ 9), since only a portion of the sulphur combines with the lead to form lead sulphide.

11. *Detection of Sulphur by Formation of a Sulphate*.—Into a nickel capsule put about .2 gramme of the substance to be tested, together with three pastilles<sup>1</sup> of caustic potash. Moisten with a few drops of water, and heat the capsule on a pipeclay triangle until the contents fuse, stirring the mixture meanwhile with an iron wire. When the fused mass appears homogeneous, drop in from a knife-point successive small portions of potassium nitrate (generally .1 or .2 gramme is sufficient). Cool, and dissolve in 3 to 5 cc. of water, again warming gently to assist solution. Turn the liquid into a test-tube, and wash out the capsule with 1 cc. of distilled water, adding the washings to the contents of the tube. Acidify with dilute hydrochloric acid (in the presence of litmus-paper), and filter. To the filtrate, add drop by drop a solution of barium chloride; in the presence of a sulphate a white precipitate of barium sulphate is produced. A blank control experiment should be performed so as to eliminate the possibility of the occurrence of sulphates as impurities in any of the reagents employed.

### Detection of Phosphorus

12. The presence of phosphorus in an organic body is indicated by the formation of a phosphate, when it is fused

<sup>1</sup> Potash pastilles or pellets, containing about .2 gramme KOH. Portions of "stick" potash may of course be substituted for these. See p. xxxii.

## DETECTION OF PHOSPHORUS

CH. I

with a mixture of potassium hydrate and nitrate. Exactly the same method of procedure is employed as is described for sulphur in a preceding section (§ II). The cool fused mass is dissolved in 3 to 5 cc. of water, with warming and subsequent filtering if necessary. Neutralize with dilute nitric acid, and test for phosphates by the following reactions.

13. To 1 cc. of the solution obtained as above is added an equal volume of 30 per cent solution of ammonium nitrate, and from 3 to 5 drops of concentrated nitric acid.

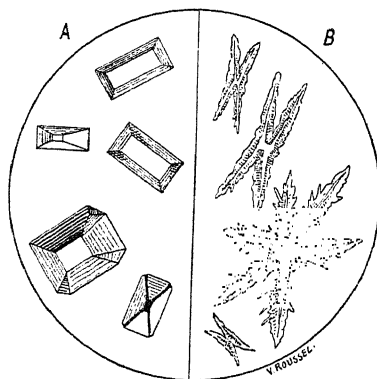


FIG. 1.—Ammonio-Magnesium Phosphate Crystals.  
(A) Due to slow crystallization. (B) Due to rapid crystallization.

Heat to boiling and add 2 cc. of a 3 per cent solution of ammonium molybdate. In the presence of phosphates a yellow precipitate of ammonium phosphomolybdate is produced.<sup>1</sup>

14. Put the remainder of the solution into a conical test-glass, add 1 cc. of a 10 per cent solution of ammonium chloride, and 1 cc. of a 10 per cent solution of magnesium sulphate; then add excess of ammonia (2 to 3 cc.). On stirring, a white crystalline precipitate of ammonio-magnesium phosphate is produced if a phosphate is present.

<sup>1</sup> The above method is preferable to the use of a stock mixture of ammonium molybdate and nitric acid, since this is very liable to undergo decomposition and become useless.

Microscopically examined, the precipitate shows characteristic crystals, which, however, differ in form, according as the crystallization has been slow or rapid (Fig. 1).

### Qualitative Analysis of Vegetable Ash

15. The method of preparation of an "ash" and the precautions necessary during incineration are described in § 61.

For its qualitative analysis, take 2 grammes of wood ash and grind them in a mortar with 20 cc. of water. Filter, and wash the residue on a filter twice with 10 cc. of water, which is then added to the original filtrate. The filtrate, which is alkaline, contains the soluble portion of the ash, and may be divided into five equal portions which are examined as follows for sulphates, chlorides, potassium, and sodium. The insoluble residue is tested for iron, manganese, calcium, phosphoric acid, and magnesium.

## SCHEME FOR SIMPLE QUALITATIVE ANALYSIS OF A VEGETABLE ASH (§§ 15-25)

The Ash is extracted with water (§ 15, § 61) and filtered.		The Filtrate (§§ 16-24) is tested for—	
<p>The Residue (§§ 22-25) is treated with 10 per cent HCl and filtered. The filtrate is treated with <math>\text{NH}_4\text{OH}</math>; a gelatinous ppt. forms; this is treated with dilute acetic acid; a portion dissolves and is filtered off.</p>	<p><i>Residue</i>.—The filter is divided in halves. One half is tested for Fe, the other for Mn.</p>		<p><i>Filtrate</i>.—</p>
	(1) IRON (§ 23)	HCl and Potassium ferrocyanide.	Blue colour or ppt.
	(2) MANGANESE (§§ 30-31) (after incineration of half filter)	Converted to Permanent acid with lead peroxide and $\text{HNO}_3$ .	Violet coloration.
	(1) CALCIUM (§ 24)	Ammonium Oxalate	White ppt.
	(2) PHOSPHORIC Acid and (3) MAGNESIUM } (§ 25)	After removal of all traces of calcium, the filtrate is treated with $\text{NH}_4\text{OH}$ .	White ppt.
	(1) SULPHATES (§ 16)	Conc. HCl and $\text{BaCl}_2$	White ppt.
	(2) CHLORIDES (§ 17)	$\text{AgNO}_3$ and $\text{NH}_4\text{OH}$ .	White ppt.; insol. $\text{HNO}_3$ ; sol. $\text{NH}_4\text{OH}$ .
	(3) POTASSIUM (§§ 18-20)	<p><math>\text{HNO}_3</math> and</p> <p>1. Perchloric acid (§ 18); or</p> <p>2. Sodium picrate (§ 19); or</p> <p>3. Platinic chloride (§ 20)</p>	<p>White ppt., slightly sol. <math>\text{H}_2\text{O}</math>; insol. in alcohol</p> <p>Yellow ppt.</p> <p>Yellow ppt.</p>
	(4) SODIUM (§ 21)	Potassium antimonate (§ 21 and note)	White ppt.

16. *Sulphates*.—The first portion of the filtrate is rendered definitely acid to litmus by the addition of a few drops of concentrated hydrochloric acid. Carbon dioxide is evolved, thereby demonstrating the presence of soluble carbonates in the ash. On the addition of barium chloride to the acid solution a white precipitate of barium sulphate indicates the presence of sulphates.

17. *Chlorides*.—The second portion, acidified with nitric acid, is treated with a dilute solution of silver nitrate. A white curdy precipitate, easily soluble in ammonia, shows the presence of chlorides.

18. *Potassium*.—Acidify the third portion with nitric acid and add perchloric acid, drop by drop. A white precipitate of potassium perchlorate, slightly soluble in water, but insoluble in alcohol, denotes the presence of potassium.

19. The presence of potassium can also be demonstrated by the following procedure. Exactly neutralize the liquid (§ 15) with dilute acetic acid, and add a few cubic centimetres of a saturated solution of sodium picrate. The liquid at first remains clear, but soon becomes turbid, owing to the precipitation of potassium picrate, which is only very slightly soluble in water. The formation of this precipitate, which is in the form of fine needle-like crystals, is facilitated by stirring.

20. A further method for ascertaining the presence of potassium is by the formation of its chloroplatinate, which forms yellow octahedral crystals which are only very slightly soluble in dilute alcohol. Put about .5 cc. of the filtrate (§ 15) into a watch-glass, and acidify with a few drops of dilute hydrochloric acid. Then add three drops of a 10 per cent solution of platinic chloride and evaporate on a water-bath to one-third of the original volume (but *not* to dryness). Add 1 cc. of alcohol; a yellow precipitate of potassium chloroplatinate forms, in the presence of potassium. The crystals have a characteristic microscopical appearance. The supernatant clear yellow fluid contains sodium chloroplatinate.

21. *Sodium*.—The remainder of the original filtrate (§ 15)

can be used for the detection of sodium. Sodium salts are not precipitated by picric or perchloric acids, nor by platinic chloride. On the other hand, the addition of potassium antimoniate, and boiling for about a minute, with subsequent cooling and stirring, produces a white precipitate of sodium pyroantimoniate. This precipitate is crystalline, and the crystals have the form of truncated octahedra.

The maximum degree of delicacy for this test is obtained

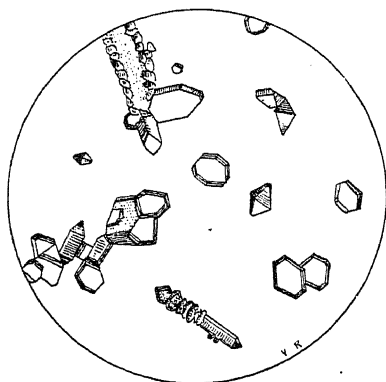


FIG. 2.—Potassium Chloroplatinate.

by adding to the liquid under examination 10 per cent of the reagent described below.<sup>1</sup> Take 5 cc. of the filtrate from the

<sup>1</sup> This reagent, discovered by Frémy, was formerly prepared by a "dry" method; it is better to prepare it in the "wet" way by oxidizing a solution of antimony hydrate in potash by potassium permanganate (Reynoso), bichromate or ferricyanide, or hydrogen peroxide (Knorre and Olschewsky). In our experience the following is a convenient method of preparation.

Into a porcelain evaporating dish put 25 grammes of caustic potash and 100 cc. of water. After it has dissolved add 10 grammes of antimony trichloride (butter of antimony), and 88-90 cc. of a 5 per cent solution of potassium permanganate. The mixture is then boiled for a few minutes, allowed to settle for a short time to see if the liquid is colourless, and filtered. If the liquid has a green colour, a little more antimony chloride must be added and the whole re-boiled and filtered. Prepared thus, the reagent gives an abundant precipitate with a solution which only contains 1 part in 1000 of sodium; even with a solution containing 1 part in 2000, a visible deposit can be obtained by rubbing the sides of the test-tube with a glass rod.



watery extract of the ash (§ 15) and add .5 cc. of the reagent. An immediate light precipitate is often produced thereby ; this must be removed by filtration. Heat the filtrate to boiling, cool, and if the required precipitate of sodium pyroantimoniate does not immediately appear, its formation can be accelerated by stirring. Should it still fail to appear (as may happen in very dilute solutions), its formation may be induced by the introduction of a trace of sodium pyroantimoniate upon a glass rod (which has been dipped first in a solution of potassium pyroantimoniate and then in a solution of sodium chloride), and vigorously stirring.

22. The filter with its insoluble residue (§ 15) is put into a conical glass and treated with 20 cc. of 10 per cent hydrochloric acid. If the previous washing with water has been properly carried out, the resulting clear liquid will be strongly acid, but should this not be the case, a further quantity of acid must be added. The filter is broken up with a glass rod and the whole filtered again, the filtrate being collected in a beaker.

23. *Iron and Manganese*.—Render the filtrate (§ 22) faintly alkaline with ammonia, added drop by drop ; a gelatinous precipitate forms which almost entirely dissolves on the addition of dilute acetic acid. On filtration, a small deposit of the phosphates of iron and manganese remains on the filter. Divide the filter with its contained residue into two. One half of the filter is used to test for iron ; the other half, after incineration, is tested for manganese (see §§ 30, 31).

For iron, the half filter is moistened with a drop of 10 per cent hydrochloric acid, and a drop of potassium ferrocyanide solution added. A blue colour indicates the presence of iron.

24. *Calcium*.—Five to ten cubic centimetres of the filtered liquid (§ 23) are brought to boiling in a small flask, and double the volume of a saturated solution of ammonium oxalate is added. Calcium, if present, forms a very fine white precipitate of calcium oxalate. In order to ensure the precipitation of all the calcium

present (which is essential for the next procedure, *e.g.* the detection of phosphoric acid and magnesium, § 25), filter off the precipitate of calcium oxalate, and test a little of the filtrate with a further quantity of ammonium oxalate. A precipitate indicates that all the calcium has not come down, and further addition of ammonium oxalate is necessary, the mixture being boiled as before.

25. *Phosphoric Acid and Magnesium*.—The filtrate as obtained above (§ 24) is cooled, made strongly alkaline with ammonia, and well stirred or shaken. A white crystalline precipitate of ammonio-magnesium phosphate shows the presence of phosphoric acid and magnesium.

### Detection of Bromine and Iodine

26. Quite appreciable amounts of iodine occur in certain seaweeds (*e.g.* common wrack—*Fucus vesiculosus*), and in certain animal tissues (thyroid gland). Iodine in seaweed is usually accompanied by traces of bromine.

*Detection in Seaweed*.—Take about two grammes of the dried seaweed, and heat it to dull redness in a small nickel capsule. Keep at about the same temperature for a minute or two, until the first incandescence has passed off, then allow it to cool. Break up the carbonized residue in a mortar, and add 5 cc. of water; grind thoroughly, filter, and wash the residue on the filter with 1-2 cc. of water.

The solution, which is alkaline and contains iodides, is slightly acidified with concentrated hydrochloric acid, in the presence of a fragment of litmus-paper. About .5 cc. of chloroform is next added, and then, *very cautiously, drop by drop*, dilute chlorine water.<sup>1</sup> The mixture is to be shaken after the addition of each drop. The chlorine displaces iodine from combination as iodide, the free iodine dissolving in the chloroform to form a pinkish violet solution. On addition of excess of chlorine the colour disappears, owing to the formation of colourless iodine chloride.

<sup>1</sup> A saturated solution of the gas in water, diluted to one-fifth its strength.

If this decolorization is carried out with sufficient care, the violet colour is seen to give place in the first instance to a rose, then to a yellow tint. This is due to free bromine, and appears when all the iodine (but not the bromine) has combined with chlorine.

27. *Iodine in the Thyroid Gland.*—Iodine exists in the thyroid gland in the form of "thyroidine." Its presence can be demonstrated by heating 2 grammes of the fresh tissue with three pastilles<sup>1</sup> of potash in a nickel capsule. Allow the mixture to fuse, then heat for a few minutes to dull redness; after cooling, extract with 5 cc. of water, warming gently if necessary. Filter, and wash the residue with about 1 cc. of water. The solution contains the iodine as iodides, which are then decomposed, and the liberated iodine recognized as shown in the preceding paragraph (§ 26).

During the heating it is necessary not to employ too high a temperature, as the iodides would thereby be volatilized and lost. On the other hand, the heating must be sufficient, otherwise tarry matters remain and tint the solution brown.

28. In these processes for the detection of iodine and bromine, chlorine-water may be replaced by other oxidizing agents. If the ferric salts (ferric chloride or iron alum) are used, the iodine alone is displaced and there is no special necessity to avoid excess of the reagent.

29. To demonstrate traces of bromine, even in the presence of iodine, the following technique may be employed (Denigès and Challe). To 5 cc. of the solution under examination add 4 drops of concentrated hydrochloric and 1 cc. of pure strong sulphuric acids. Mix thoroughly, and add 1 cc. of a decolorized solution of fuchsin<sup>2</sup> and 4 drops of a 10 per cent solution of potassium bichromate. To the still warm liquid (or to the liquid reheated if necessary to

<sup>1</sup> See note, p. xxxii.

<sup>2</sup> To prepare this reagent add 5 cc. of concentrated sulphuric acid to 95 cc. of water, allow to cool, and add 10 cc. of a fresh 1 per cent solution of fuchsin.

The mixture gradually decolorizes, and after about an hour it is ready for use.

50° C.) add 1 to 2 cc. of chloroform, and shake well for about a minute. If bromine is present, a coloured bromine derivative of fuchsin is formed, which tinges the chloroform a violet red.

This reaction is available for the detection of bromine in water samples; it is sufficiently sensitive to detect 1 mgr. of bromine in a litre of water.

### Detection of Manganese

30. For demonstration purposes, vegetable material known to be rich in manganese is the most suitable; tea answers admirably. Gently incinerate about .5 gramme of tea leaves in a porcelain capsule; allow it to cool, and drop the residue (which should be grey or greyish-brown) into a test-tube, adding a pinch of lead peroxide and 5 cc. of nitric acid. Five cubic centimetres of water are next added, and the mixture boiled for two or three minutes. Allow it to settle, when the supernatant liquid will be coloured violet owing to the formation of permanganic acid, thereby indicating the presence of manganese in the original material.

The incineration must be complete; if a small amount of carbon remains, the permanganic acid is reduced, and the characteristic colour is either not obtained at all or is evanescent.

The presence of chlorides materially diminishes the sensitiveness of the reaction. When they are present in any quantity, the ash should be placed on a filter, extracted with water, and a few drops of warm alcohol added. The filter and residue are then incinerated, and the ash is treated as described previously (*e.g.* lead oxide, nitric acid, etc.).

31. Manganese can also be detected by the "dry" method. For this, a red-hot platinum loop is dipped into a fusion-mixture.<sup>1</sup> On withdrawal and subsequent reheating in the Bunsen flame, an adherent colourless bead is formed. Plunge the hot fused bead into the ash under

<sup>1</sup> Sodium carbonate (anhydrous) 9 parts, potassium nitrate 1 part.

examination, and heat for a moment or two in the outer Bunsen flame. The presence of manganese is shown by a green coloration of the bead.

### Detection of Iron in the Blood

32. The red blood corpuscles of vertebrates contain iron in the form of hæmoglobin. Its presence is easily demonstrated as follows. A drop of defibrinated blood is placed in a small porcelain capsule, gently heated and then carefully incinerated. The reddish-brown residue is dissolved in a few drops of concentrated hydrochloric acid, with the aid of slight heat if necessary. Dilute with 2-3 cc. of water, and add a few drops of potassium ferrocyanide ; the resulting blue colour shows the presence of iron.

### Detection of Copper in Snail's Blood

33. In certain invertebrates (*e.g.* Mollusca and Crustacea) copper is found in the blood instead of iron. It may be demonstrated in snail's blood as follows. Take a live snail, and fracture the shell at some distance from its mouth, about the situation of the first turn of the spiral ; cautiously enlarge the opening with forceps, taking care not to injure subjacent structures. The heart will be recognized by its rhythmic pulsations ; open the pericardium with fine-pointed scissors and collect the blood. It is well to make the animal contract into its shell, as the quantity of blood obtained is thereby increased. The blood is at first colourless, but rapidly becomes blue on exposure to air. The specimen is put into a small porcelain capsule with a few drops of concentrated sulphuric acid ; the coagulum produced is carefully heated until it becomes first brown and then black. When carbonization is complete, it is allowed to cool, then rubbed up with a few cubic centimetres of water, filtered, and the residue washed with a few drops of water. The filtrate contains alkali phosphates, together

with a trace of copper, which latter can be detected by the addition of formaldoxime,<sup>1</sup> when a violet colour appears.

34. The greater part of the copper is, however, contained in the residue. The filter and residue are carefully warmed until dry, and then incinerated. To the cooled white or grey ash, 5 drops of pure hydrochloric acid are added, and the mixture is then evaporated to dryness. The residue is treated with a few drops of strong ammonia and filtered in order to separate traces of iron which are always present and would mask the subsequent reactions. The filtrate, which has a faint blue colour, is acidified with concentrated hydrochloric acid, and potassium ferrocyanide solution added, drop by drop. A reddish colour or precipitate shows the presence of copper. It is absolutely essential to remove all traces of iron, as the blue colour-reaction of iron and potassium ferrocyanide quite masks that of copper.

### Detection of Boron in Wine

35. Boron occurs naturally in very small amounts in various tissues, and can be found in their ash by the following method (G. Bertrand and H. Agulhon). It is easiest found in vegetable matters and especially in wine.

*Detection by Means of Turmeric Paper.*—Evaporate to dryness in a capsule on a water-bath 20 cc. of wine previously rendered faintly alkaline; calcine the residue just below a dull red heat, then after cooling, faintly acidify the ashes (which contain the boron as borates) with 10 per cent hydrochloric acid, and filter, collecting the filtrate in a small evaporating basin (about 3 cm. in diameter) furnished with a lip. Take a long narrow strip of turmeric paper,<sup>2</sup> 4 cm. long by 3 mm. wide. Immerse one end in the liquid and allow the other end to hang free over the lip of the basin

<sup>1</sup> The solution of formaldoxime is prepared by dissolving 1 part of crystallized hydroxylamine hydrochloride in a mixture of 1 part commercial formalin and 1 part water. Solution is assisted by gentle warming.

<sup>2</sup> Turmeric paper is made by soaking filter-paper in tincture of turmeric. For the tincture 1 part of powdered turmeric is boiled with 25 parts of alcohol.

for about 1 cm.; the intermediate part of the strip is to remain in contact with the inner wall of the basin. Cover with a watch-glass and allow to remain for some hours, or even for a whole day. In the presence of boric acid, the free end of the paper is coloured bright red, and turns blue when touched with dilute ammonia (*i.e.* strong ammonia diluted to one-tenth its strength).

36. *Detection as Boron Fluoride.*—For this very delicate test it is desirable to separate the boron from the other constituents of the ash, as methyl borate, which can be isolated by distillation. After saponification of this ester, the resulting boric acid is converted into boron fluoride by means of calcium fluoride and sulphuric acid. Boron fluoride is a gas and can be recognized by the green colour which it imparts to the Bunsen flame. Proceed as follows. The ash prepared as above (§ 35) is placed in a little flask of 90 cc. capacity, together with 5 cc. of phosphoric acid,<sup>1</sup> and 20 cc. of pure methyl alcohol. Fit the flask to a condenser and distil on a water-bath. The distillate is collected in a small flask containing *two drops* of a 10 per cent solution of pure caustic soda. It is essential to take no more soda solution than this, in order to avoid undue dilution of the sodium borate produced. When no more distillate passes over, add another 5 cc. of methyl alcohol to the contents of the distilling flask, and distil again. Ascertain that the distillate collected is alkaline (from the soda first introduced into the receiver), and heat the distillate on the water-bath with a reflux condenser for ten minutes to saponify the methyl borate. Then transfer the liquid to a small capsule and evaporate to dryness on a water-bath.

The residue is thoroughly ground up with double the quantity of calcium fluoride (finely powdered in an agate mortar), and the mixture made into a thick paste with pure concentrated sulphuric acid. Take a little of this paste on a platinum loop and hold it within about 2 mm. of the base of a Bunsen flame, the outer zone of which then acquires

<sup>1</sup> Prepared by treating 1 part of phosphoric anhydride with 1 part of water.

the characteristic green colour. It is advisable not to work with a large flame, so as to avoid draught and the consequent too rapid dispersal of the boron fluoride. Three equidistant green bands are seen on spectroscopic examination.

### Estimation of Nitrogen (Kjeldahl's Method)

37. This method depends upon the destruction of organic matter by boiling with concentrated sulphuric acid, and the subsequent production of ammonia. The titration then resolves itself into a quantitative estimation of ammonia.

38. *Treatment of the Substance with Sulphuric Acid.*—Weigh out accurately a small quantity of the material to be examined; the amount needed in individual cases varies, but in general, such an amount is taken that it contains 20-40 mgr. of nitrogen. For example, if barley or malt is being studied, take 1-2 grammes; if albumin or gelatine, take .2-.3 gramme. Put this into a round-bottomed flask (preferably with a long neck) of about 90 cc. capacity; add exactly 10 cc. of pure concentrated sulphuric acid and a drop of mercury about the size of a hempseed.

Heat first of all gently and then to boiling, in a fume chamber. The heating must be regulated so that the condensed acid fumes carry back with them any particles that may be accidentally spurted up. Too violent boiling must be avoided, as this gives rise to excess of disagreeable acid fumes and renders the contents of the flask too concentrated. As the heating progresses the liquid becomes first brown, then yellow, and is finally decolorized; at this stage, stop the boiling, and after partial cooling add *very small quantities* of powdered potassium permanganate, until a permanent green or violet colour is produced; excess of permanganate must, however, be carefully avoided. By this procedure the organic matter is entirely decomposed; the carbon is oxidized to carbon dioxide, while the nitrogen in the form of ammonia unites with the excess of acid, an ammonio-mercuric sulphate being formed which crystallizes as white needles on cooling.



39. *Distillation and Titration of the Ammonia formed.*—The apparatus (Fig. 3) consists essentially of a flask connected with an ascending spiral cooling tube connected with a water-jacketed condenser. The ascending part of the tube acts as a separator, in which the larger part of the steam condenses and is returned to the distilling flask, while the portions of distillate rich in ammonia pass into the condenser and are collected.

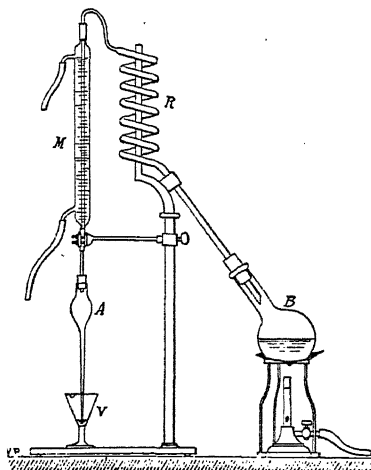


FIG. 3.—Schloesing-Aubin Apparatus for Kjeldahl Estimations.

B. Distilling flask. R. Spiral cooling tube. M. Water-jacketed condenser.  
A. Delivery tube, finished with bulb and drawn out at its lower end.

40. To conduct the distillation the acid liquid (§ 38) is diluted with 60 to 80 cc. of water. The resulting rise of temperature facilitates the solution of the crystals, which should be complete. The solution obtained is placed in a flask of 500 cc. capacity; wash out the flask in which the decomposition was effected two or three times with distilled water and add the washings to the contents of the 500 cc. flask. It is next necessary to precipitate the mercury, since the ammonio-mercuric salt is only attacked with difficulty by soda; for this purpose an energetic reducing agent such as sodium hypophosphite is used (Maquenne). Add 1-2

grammes of this salt to the still warm liquid ; it produces a white precipitate which becomes black (reduction to metallic mercury). Should the liquid have become cold, the reaction is slow, and must be hastened by gentle heating. Make sure that the precipitation of the mercury is complete, by adding a further pinch of the hypophosphite and noting that no further precipitate is formed. Next, add enough water to bring the total volume to 200 cc. ; and then, in a single dose, add 40 cc. of strong soda solution (36° B.).<sup>1</sup>

Immediately connect up the flask with the rest of the apparatus, and then distil. The drawn-out extremity of the bulb A is placed beneath the surface of 5 cc. of water in the glass V, the liquid being coloured with a drop of heli-anthin. (*Do not forget to turn on the water in the condenser.*)

When the indicator turns yellow, sufficient N/5 sulphuric acid is run in to bring it exactly to a rose colour. When no further change occurs all the ammonia has passed over, and the distillation may be stopped. The bulb A must be detached from the apparatus to avoid back suction on cooling.

If the acid is exactly N/5, each cubic centimetre used corresponds to .0028 gramme of nitrogen. If  $p$  be the weight of substance taken, and  $n$  the number of cubic centimetres of acid used, the percentage of nitrogen is

$$.0028 \times n \times 100$$

The method described is suitable for the estimation of nitrogen in animal or vegetable tissues, etc. It is not applicable where the nitrogen exists as nitrates (nor in their presence) ; and it is not recommended for such substances as alkaloids, where the nitrogen is contained in a cyclic nucleus.

### Preparation of "Normal" Sulphuric Acid

41. Speaking generally, a "normal" solution of an acid is a solution which contains per litre a quantity of acid

<sup>1</sup> *i.e.* enough to make the reaction strongly alkaline. For correspondence between "degrees Beaumé" and specific gravity see note, p. xxxii, and Tables, pp. 322-328.

corresponding to one atom of hydrogen replaceable by a metal. If the acid used is monobasic (hydrochloric, acetic, etc.), one gramme-molecule of acid per litre is taken; if it is dibasic (sulphuric, oxalic, etc.) half a gramme-molecule, and so on. In the same way, a "normal" solution of an alkali is one which exactly combines with its own volume of "normal" acid.

42. To prepare a normal solution of an acid, the most simple and practical method consists in taking an accurately weighed sample of some ammonium salt and distilling it with soda in the apparatus described in § 39. The titre of the acid can be calculated from the amount necessary to combine with the liberated ammonia. In practice the most convenient ammonium salt to use is the oxalate  $(\text{NH}_4)_2(\text{COO})_2 + \text{H}_2\text{O}$ , since it is easily obtained pure, and is neither efflorescent nor deliquescent.

43. The method is as follows. Weigh out .710 gramme of the salt and introduce it into the distilling flask of the apparatus (§ 39), with 150 cc. of water and a few cubic centimetres of sodium hydrate solution; distil, and collect the distillate in a little water (4-5 cc.) tinged with helianthin. A single drop of the indicator is sufficient; any excess must be avoided if the maximum degree of delicacy is to be attained. As soon as ammonia distils over, the indicator becomes yellow. As the distillation proceeds, the acid<sup>1</sup> to be titrated is run in from a graduated burette until the indicator reassumes its original rose colour. Every time the yellow reappears a little more acid is run in. Towards the end of the operation the addition must be made very

<sup>1</sup> The acid for titration is made by adding 52-53 grammes of concentrated sulphuric acid (166° B., see p. xxxii) to 700-800 cc. of water in a graduated litre flask. The water should be put into the flask first, and the acid added little by little with constant shaking. The whole is cooled and the volume made up to 1 litre. By this procedure not only is the small amount of water which the acid may contain allowed for, but a more concentrated liquid than "normal" is obtained, which allows of suitable dilution. Theoretically, of course, 49 grammes of acid should be taken. If it is desired not to *weigh* the acid, 30 cc. of acid may be taken in a pipette, and the total volume made up to 1050 cc. If the acid is weighed or measured do not forget to rinse out the containing vessel with water, which is added to the contents of the measuring flask.

cautiously drop by drop, in order to avoid overshooting the mark. The titration is ended when the rose no longer changes to yellow. The quantity of ammonium oxalate employed (·710 gramme) corresponds to exactly 10 cc. of "normal" acid. Hence if  $n$  cc. of acid are run in, it is evident that these  $n$  cc. must be diluted to 10 cc., to give "normal" acid. A volume  $v$  of acid must have a volume of water equal to  $\frac{v}{n}(10 - n)$  added, to bring it to "normal."

44. Generally speaking, "normal" sulphuric acid is employed for titrating alkaline solutions. It will be remembered that since sulphuric acid is dibasic, one litre of the "normal" acid will contain *half* a gramme-molecule of  $\text{H}_2\text{SO}_4$  (*i.e.* 49 grammes), corresponding to one atom of replaceable hydrogen. This amount is capable of neutralizing *one* gramme-molecule of monovalent alkali (soda, ammonia, potash), or *half* a gramme-molecule of dibasic alkali (lime, baryta, potassium carbonate, etc.).

It follows that if we dissolve one gramme-molecule of monovalent alkali, or one half gramme-molecule of divalent alkali, in one litre of water, these solutions will correspond exactly to "normal" sulphuric acid, volume for volume. Put in another and very practical way, 1 cc. of normal (N/1) sulphuric acid corresponds also to

gram.	gram.
·056 of KOH	·040 of NaOH
·017 „ $\text{NH}_3$	·028 „ CaO
·0765 „ BaO	·069 „ $\text{K}_2\text{CO}_3$ .

In calculating the value of a given titration, simply multiply the number of cubic centimetres of acid employed for neutralization by the factor corresponding to the alkali in question in order to find the amount of alkali present. Thus, if in titrating a sample of caustic soda,  $n$  cc. of "normal" acid were used, the amount of soda present would be  $n \times 0.040$  gramme.

### Estimation of the Alkalinity of Ash

45. Weigh out 5 grammes of sawdust and carefully incinerate it in a crucible with a muffle, taking care not to go beyond a dull red heat. When the ash is nearly white, allow it to cool and put it in a beaker, adding from 5 to 10 cc. of water and one drop of helianthin. Next run in a slight excess of decinormal (N/10) hydrochloric acid (from 15 to 20 cc.) until a permanent rose colour is obtained; warm and stir gently to assist the reaction. Then run in decinormal soda until the yellow tint is just reached. The quantity of acid used, *minus* the quantity of soda, corresponds to the alkalinity of the ash. It can be expressed, for example, in terms of potassium carbonate. If the ash contains free carbon, the helianthin will be absorbed and the solution decolorized, thus rendering the indicator useless.

### Colorimetric Estimation of Manganese

46. Titration of the amount of manganese can be carried out colorimetrically, by oxidizing it to permanganic acid.

*Preparation of the Ash.*—The dried tissue or extract is incinerated at a dull red heat till all carbon has disappeared. Allow it to cool, add slight excess of concentrated hydrochloric acid, and warm on a water-bath. When the liquid is colourless add a little sulphuric acid to decompose any chlorides present, then evaporate to dryness and re-incinerate at a dull red heat. If the heating has been excessive, the residue must be moistened with a drop of hydrochloric acid and warmed for a few minutes; then a little dilute sulphuric acid is added and the whole evaporated till white fumes appear.

47. *Oxidation of Manganese.*—The ashes are put in a test-tube furnished with a 10 cc. mark, and dissolved in 2-3 cc. of dilute nitric acid (acid at 36° B.<sup>1</sup> diluted with three times its volume of water); from 2 to 5 drops of a

<sup>1</sup> See Tables, pp. 322-328.

10 per cent solution of silver nitrate, and .2-.3 gramme of powdered potassium persulphate are next added, the whole made up to 10 cc. with distilled water and gently warmed. A rose-violet colour shortly appears, varying in depth according to the quantity of manganese present. The mixture is gradually brought to boiling to decompose the excess of persulphate, and the boiling stopped when all evolution of oxygen has ceased; the tube is then covered and allowed to cool.

48. *Colorimetric Estimation of Manganese.*—First prepare a solution containing 4.054 grammes of manganese sulphate in one litre of water. The salt should be well crystallized and not efflorescent. Dilute this solution, which contains 1 mgr. of manganese per cc., one hundred times (*i.e.* 10 cc. in 1 litre of water). Then take a series of perfectly clean<sup>1</sup> test-tubes, and put into them exactly measured quantities of the dilute manganese sulphate solution, for example, 5 cc., 2 cc., 1 cc., .5 cc., .2 cc., .1 cc. These are made up to 10 cc. each with distilled water, giving quantities of manganese respectively equal to  $\frac{1}{20}$ ,  $\frac{1}{50}$ ,  $\frac{1}{100}$ ,  $\frac{1}{200}$ ,  $\frac{1}{500}$ ,  $\frac{1}{1000}$  milli-gramme of manganese. Oxidize with potassium persulphate as above; a colour series is thus obtained with which the tint of the experimental tube can be compared. By taking a sufficient number of standards a close approximation to the amount of manganese present can be obtained.

### Estimation of Iron in the Blood

49. Iron can easily be estimated as a ferrous salt by titration with potassium permanganate. From 3 to 5 cc. of blood are taken and exactly weighed in a tared capsule. This is then gently heated till a clot forms and then to dryness. The residue is next incinerated, taking care that none is lost by bubbling at the commencement of the operation. The complete combustion of all carbon present is ensured by adding a few small crystals of ammonium nitrate, and the

<sup>1</sup> By boiling with a little nitric acid and thoroughly washing with distilled water.

residue is then dissolved in a few drops of concentrated hydrochloric acid. It is then evaporated to dryness, a few drops of sulphuric acid are added to decompose chlorides, and the whole reheated until white fumes are evolved.

To the residue so obtained a little concentrated sulphuric acid is added, and the whole is re-dissolved in a few cubic centimetres of 10 per cent sulphuric acid. The solution is transferred to an Erlenmeyer flask, a piece of granulated zinc is added, and the flask closed with a cork through which passes a glass tube with a narrow outlet. The liberated hydrogen reduces ferric to ferrous salts; after 15-20 minutes, the reduction is complete and the liquid colourless.

The liquid is now transferred to another flask, by decantation, the original flask being washed out three times with a little boiled distilled water. Care must be taken that no particle of zinc passes over. It is then titrated with a solution of potassium permanganate (·5 gm. per litre) until a faint permanent pink is obtained.

The solution of potassium permanganate itself can be easily titrated by means of a solution of iron-alum,  $\text{Fe}_2(\text{SO}_4)_3 + (\text{NH}_4)_2\text{SO}_4 + 24\text{H}_2\text{O}$ . Exactly 8·6 grammes of this salt are weighed out, put into a graduated 1-litre flask, and dissolved in a little water to which a few drops of sulphuric acid have been added. The solution is made up to one litre and well mixed.

For the titration 10 cc. of this solution (which contains 1 mgr. of iron per cc.) are put into a flask, with 1 cc. of sulphuric acid and a piece of granulated zinc, the resulting liquid being decanted and titrated as above.

## CHAPTER II

### CHIEF INCOMBUSTIBLE GROUPS

Nitrates—Nitrites—Thiocyanates—Water—Chlorides—Phosphates

#### Detection of Nitrates

50. NITRATES are mainly formed in the soil by fermentation ; thence they are partly absorbed by the roots of plants, and partly washed into streams and rivers by rain.

*Detection of Nitrates in Soil and Water.*—In the case of soil, 10 grammes of soil are mixed with 10 cc. of water, the mixture is filtered, and a small quantity of the filtrate evaporated to dryness on the water-bath. In the case of waters, 1-10 cc. are similarly evaporated, the residues being treated as follows in either case.

51. After complete cooling, the residue is mixed with a few drops of pure sulphuric acid ; a few milligrammes of ferrous sulphate are next added (a small crystal crushed between two leaves of paper suffices), and the mixture stirred. In the presence of nitrates a rose colour makes its appearance, which is due to reduction of nitric acid to nitric oxide by ferrous sulphate, and the subsequent combination of this gas with the excess of ferrous sulphate.

52. In place of this characteristic reaction, the following is often employed. A drop of diphenylamine<sup>1</sup> reagent is allowed to fall upon the residue from evaporation of the

<sup>1</sup> Diphenylamine reagent is prepared by mixing 100 cc. of pure  $H_2SO_4$ , 5 cc. of 5 per cent solution of diphenylamine sulphate, and 5 cc. of 10 per cent HCl.



sample of water or filtrate, when a strong blue colour is given if nitrates (or nitrites) are present.

53. A further test for the presence of nitrates is to moisten the residue obtained as in § 50 with one or two drops of concentrated sulphuric acid, and then to add a few milligrammes of brucine. A red colour is produced which changes first to orange, then to yellow.

54. *Detection of Nitrates in Plants.*—Nitrates are easily detected in fresh plants provided they are examined soon after being gathered. A small quantity of the plant (nettle, clover, etc.) is crushed in a mortar and a drop of the juice removed with a glass rod. To this are applied the tests described in the preceding paragraphs.

Generally it is sufficient to rub the fresh section of a plant stalk upon a white porcelain capsule, and to apply the tests directly.

### Detection of Nitrites

55. Nitrites can be detected in samples of soil or water by the following reactions :

Make some fresh starch paste, by boiling a fragment of starch the size of a pin's head with 6-8 cc. of water ; after cooling add 1 cc. of a fresh solution of potassium iodide (or a small crystal of the salt), and acidulate with a few drops of acetic acid.

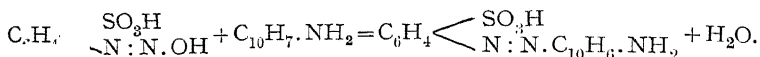
The mixture should remain colourless ; if prepared with a stale solution of iodide already tinged yellow by free iodine, it will be blue and useless. If to this colourless mixture there be added a drop of a very dilute solution of a nitrite (washing of soil, or a sample of water concentrated by evaporation on the water-bath), an intense blue colour appears due to the combination of starch with iodine liberated by nitrous acid.

56. The foregoing reaction is given by the majority of oxidizing substances ; the following is specific for nitrites.

Into a test-tube put 5 cc. of a filtered 1 per cent solution of  $\beta$ -naphthylamine, acidified with 10 per cent of acetic acid, and 5 cc. of a 1 per cent solution of sulphanilic acid ;

no colour results from the admixture, but in the presence of a trace of nitrite a rose colour appears (Griess' reaction).

The nitrous acid liberated by the acetic acid in the reagent reacts with the sulphanilic acid to produce a diazo-compound, which in turn combines with the naphthylamine to form a red compound according to the following equation :



### Detection of Thiocyanates

57. Thiocyanates often exist in small quantities in saliva, gastric juice, and urine. In saliva they can be recognized by the following reactions.

A few cubic centimetres of saliva are rendered definitely acid with dilute hydrochloric acid ; a very dilute solution of ferric chloride is next added drop by drop ; an orange or red coloration indicates the presence of thiocyanates.

58. Make freshly about 5 cc. of dilute starch paste and add a trace of potassium iodate (a fragment about the size of two pin-heads). Acidulate the mixture with a little dilute hydrochloric acid, no coloration should occur ; but on the addition of a few cubic centimetres of saliva the solution becomes blue, due to the formation of iodide of starch : thiocyanic acid reacts with iodic acid, and liberates iodine.

### Estimation of Water

59. Substances of animal or vegetable origin frequently contain large quantities of water. In order to estimate these, a certain quantity of the material under investigation is accurately weighed, then dried and weighed again. The difference between the original and final weighings gives the amount of water lost. The water-content of such substances is expressed as a percentage ; thus if  $W$  and  $w$  represent the weights of a substance before and after desiccation respectively, the percentage of moisture originally present is obviously

$$\frac{W - w}{W} \times 100.$$

On account of the instability of animal and vegetable matter at elevated temperatures, the temperature at which desiccation is effected must not exceed  $120^{\circ}$ . The material is placed in a small very light flask, and put into an oven regulated for, *e.g.*,  $100^{\circ}$ . After a few hours the flask is removed from the oven, corked, and weighed. (It is sometimes convenient to use a second flask as a partial counterpoise, suitable adjustments of weights being made.) The experimental flask, with its cork removed, is replaced in the oven and left for another hour or two. It is then re-weighed, and if the weight is found to be the same as when first weighed, the contents may be regarded as dry. Should this not be the case, it is replaced in the oven until no further loss of weight is found between two consecutive readings. This procedure is known as "Drying to constant weight."

In place of using glass flasks for the desiccation, it may be carried out in a porcelain "boat" such as is used in combustions. The boat on removal from the oven is then transferred to a corked tube for weighing.

60. With certain bodies, especially starchy substances, a state of equilibrium is obtained with the moisture of the atmosphere, so that their degree of desiccation is a function of its hygrometric condition. In other words, the desiccation is relative and never complete (Maquenne), and in such cases it is necessary to carry out the drying in a current of dry gas (air, hydrogen, or carbon-dioxide). The substance is put in a boat placed in a horizontal glass tube, which in turn is placed in a suitable oven, and the gas selected is allowed to pass over the boat and its contents, after bubbling slowly through strong sulphuric acid at the rate of about one litre per hour. Under these conditions desiccation is complete in one hour at  $120^{\circ}$ , or in two hours at  $100^{\circ}$ . The little boat is furnished with a ring at one of its extremities, and withdrawn with the aid of a hooked metal wire after the desired time. The weighing is carried out in a corked tube as before, drying being repeated until constant weight is attained.

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### Incineration

61. Substances of animal or vegetable origin when submitted to incineration leave a residue of mineral matter known as the *ash*. This ash, of very complex composition, contains usually appreciable amounts of salts of the alkali metals (chlorides, phosphates) which are more or less fusible. If the heating be excessive fusion occurs, the fused salts envelop parts of the carbon and so prevent its oxidation; on the other hand, some of the salts (chlorides, bromides, iodides) are volatile at a red heat. For these reasons, incineration must always be carried out at the lowest possible temperature; practically, it may be said that a dull red heat must not be exceeded. Under these conditions, the oxidation of carbon resulting from decomposition of the organic matter takes place with a maximum of rapidity; it is only when the incinerated material is exceptionally rich in alkali salts that the slightest difficulty arises in obtaining a complete decarbonization by simple direct heat.

In such cases, instead of maintaining the residue indefinitely at a temperature which threatens loss of some of the mineral constituents, when the carbon has disappeared as far as seems likely, the residue should be allowed to cool and then be treated with a little water. The alkali salts which envelop and protect the particles of carbon are thus dissolved; the liquid and such insoluble residue as remain are put on a small filter and washed with a little water until soluble salts are practically removed; this can be demonstrated by evaporating a drop of the filtrate on the water-bath and observing that there is no residue or almost none. The filter is now transferred to the incinerating capsule, gently dried, and reheated. There is no danger to be apprehended now, either from fusion of alkali salts or from volatilization, so that the incineration can be conducted at a bright red heat. The carbon rapidly disappears, leaving only a residue of insoluble salts.

If an estimate of the total ash is required, the washings obtained as described above are emptied into the incinerating

capsule, and the whole evaporated to dryness upon the water-bath ; a brief heating at *dull* redness completes the operation, and crucible and contents are ready for cooling and weighing.

The source of heat for an incineration may be either a gas-jet, an alcohol lamp, or a furnace fitted with a muffle. The gas-jet is simple, but has the disadvantage of introducing into the ash traces of sulphuric acid derived from small quantities of sulphur compounds in the gas itself. It may also in the case of ordinary muffle-furnaces, where the fittings are of copper, similarly introduce traces of copper.

When the presence or exact determination of small amounts of sulphur are the subject of investigation, the use of the spirit lamp is especially indicated. If, on the other hand, traces of copper are sought, the muffle heated with wood charcoal is the most suitable.

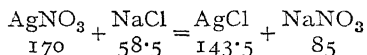
For the purpose of "ashing," platinum capsules are commonly used ; porcelain capsules may also be employed, but only when the working temperature is insufficient to lead to fusion of the alkali salts ; if this occurs, action may take place between them and the silicates contained in the glaze of the porcelain.

### Estimation of Chlorides in Urine

62. Five cubic centimetres of urine are taken in a small porcelain or nickel capsule, a few decigrammes of pure sodium carbonate added, and the whole evaporated to dryness over a small flame without ebullition. The residue is incinerated, care being taken to avoid spurting, and the temperature never being allowed to exceed a dull red, so as to avoid volatilization of chlorides. When incineration is complete, the whole is allowed to cool and 5 to 10 cc. of warm water added. After stirring and allowing to settle for a moment, the colour of the supernatant liquid is observed ; it should be colourless—if a yellow tint persists it indicates incomplete destruction of carbon, and it is necessary again to evaporate to dryness and heat to dull redness.

The colourless liquid is passed through a filter, and the residue washed with warm water so as to collect all the chlorides present, in the filtrate.

63. *Estimation of Chlorides by Mohr's Method.*—Neutralize the filtrate obtained as above with very dilute nitric acid added drop by drop; if the neutral point has been slightly passed, a small pinch of pure powdered calcium carbonate should be added. Then add three or four drops of a 10 per cent solution of neutral potassium chromate to serve as indicator, and run in a decinormal solution of silver nitrate<sup>1</sup> from a burette, stirring continuously meanwhile. The silver nitrate solution is added until a faint permanent red colour is produced; this colour is due to the presence of silver chromate, which is not formed until all the chlorine present (as chloride) has been converted into silver chloride. From the equation



a little calculation will show that each cubic centimetre of silver solution corresponds to 3.55 mgr. of chlorine or to 5.85 mgr. of sodium chloride.

If numerous samples of urine have to be examined, calculations may be simplified by making the silver solution contain 14.530 grammes of silver nitrate per litre, corresponding to 1 gramme of sodium chloride per litre.

This method may be applied directly to the urine without previous incineration, but the results are inexact; and if it is desired to avoid incineration the following method is preferable.

64. *Estimation of Chlorides by Volhard's Method.*—Ten cubic centimetres of urine are placed in a graduated 100 cc. flask, with enough nitric acid to render the mixture definitely acid to litmus (1 to 2 cc.). Decinormal silver nitrate

<sup>1</sup> This is made by dissolving silver nitrate in water (17 grammes in 1 litre). The salt should be well crystallized and free from any excess of acid, or still better should be fused in a little porcelain crucible, but at a temperature sufficiently low to avoid any decomposition (shown by blackening).

solution is next added in quantities of 5 cc. at a time, until it appears to be in excess, which is indicated thus. After each addition the mixture is well shaken, so as to assist as far as possible the precipitation of silver chloride. As long as chlorides remain in solution, complete clearing does not take place readily; when, however, there is excess of silver solution, a curdy precipitate results after shaking, and leaves an almost clear supernatant liquid.

The whole is now made up to 100 cc. with distilled water, shaken and filtered.

It is next necessary to estimate the excess of silver contained in the liquid. Fifty cubic centimetres are put into a test-glass; 2 to 3 cc. of a 5 per cent solution of ferric sulphate (or of iron-alum) are added, and then drop by drop from a burette a decinormal solution of potassium thiocyanate.<sup>1</sup> First of all a white precipitate of silver thiocyanate forms, then when all the silver has been precipitated the red colour due to the iron salt appears. At this point the reaction is stopped. The number of cc. of thiocyanate solution employed, multiplied by 2, indicates the quantity of decinormal silver solution remaining in excess of that used to precipitate the chlorides in 10 cc. of urine. If  $V$  be the total number of cubic centimetres of  $N/10$  silver nitrate used for 10 cc. of urine,  $v$  the excess remaining after all the chlorides have been precipitated, and  $n$  the number of cc. of thiocyanate solution used, then the sodium-chloride content of the urine in grammes per litre is

$$(V - 2n) \times .00585 \times 100.$$

65. *Gravimetric Estimation of Chlorides.*—This estimation is based upon the precipitation of silver chloride, which is then washed, dried, and weighed.

<sup>1</sup> To prepare this solution 10.12 grammes of potassium thiocyanate are dissolved in 1 litre of water and the whole thoroughly mixed. It is titrated by running it drop by drop from a burette into a beaker containing 10 cc. of  $N/10$  silver nitrate and 20 cc. of water, to which are added 2 or 3 cc. of the ferric sulphate solution and 10 drops of nitric acid. The mixture is constantly stirred, and the volume ( $v$ ) necessary to produce a permanent red colour is noted. To make the thiocyanate solution exactly normal,

add to each litre made as above a quantity of water equal to  $\frac{10-v}{v} \times 1000$ .

The ash from 10 cc. of urine is extracted with water as described in § 62, and the extraction must be continued until a drop of the filtered liquid collected in a watch-glass does not give the slightest turbidity with a solution of silver nitrate. The whole filtrate from the extracted ash is put into an Erlenmeyer flask and acidified with 1 to 2 cc. of nitric acid. Then a 10 per cent solution of silver nitrate is added drop by drop, with continuous shaking of the mixture as long as a precipitate is formed ; this precipitate falls and collects easily as soon as there is an excess of silver nitrate.

Warm on the water-bath for a few minutes until the precipitate has settled, and decant the liquid upon a filter previously moistened and supported by a 60° funnel. The precipitate remaining in the flask is shaken up with warm water acidified with a drop of nitric acid, allowed to settle, and the supernatant liquid again decanted. This washing by decantation is repeated two or three times, and then the whole precipitate is washed on to the filter with the assistance of a jet from the wash-bottle. Wash again on the filter with warm water, until the filtrate gives no turbidity with hydrochloric acid, and leave to drain. The funnel, filter, and contents are then dried in the air-bath at 100°-110°.

After drying, the filter is removed from the funnel and placed point-upwards upon a sheet of glazed paper (black for preference). By gentle pressure the precipitate is detached from the filter-paper, which is now folded and placed in a small tared capsule, while the precipitate resting on the black glazed paper is covered with a large glass funnel to avoid risk of loss.

The capsule is first gently heated to carbonize the filter, and then more strongly to burn it ; it is next allowed to cool, the ash is moistened with a drop of nitric acid and then evaporated gently to dryness. A drop of hydrochloric acid is next added and the evaporation repeated. By this means any particles of metallic silver reduced by the action of the carbon formed during incineration of the paper are again



converted into silver chloride. The main bulk of the precipitate is now introduced into the capsule and the whole heated until the silver chloride just commences to fuse round the edges. It is then allowed to cool and finally weighed.

The weight of silver chloride multiplied by 0.4077 gives the quantity of sodium chloride contained in 10 cc. of urine.

### Behaviour of Phosphoric Acid and of Phosphates in the Presence of Different Indicators

66. Phosphoric acid,  $\text{PO}(\text{OH})_3$ , is a tribasic acid in which the acid functions react differently with different indicators. The first, comparable as regards activity with that of sulphuric or hydrochloric acids, acts upon all indicators, litmus, helianthin, and phenolphthalein. When this is saturated, the second behaves as a feeble acid like carbonic acid; it acts on (alkaline) phenolphthalein, more definitely on litmus, and most of all on helianthin. As regards the third it possesses such feebly acid properties that it is unable to displace the indicators mentioned when they are combined as alkaline salts.

These characters may be employed to determine whether a given solution contains phosphoric acid in a free or more or less combined state. They may also be applied to the titration of this acid or of its sodium, potassium, or ammonium salts.

67. As an example the following may be given. Into three glasses are put 10 cc. of phosphoric acid solution containing 1 gramme-molecule of the acid per litre (98 grammes, or practically 158 grammes of the commercial acid at  $45^\circ \text{B.}$ ). To the first glass add a drop of orange III. (or helianthin), to the second 1 drop of an alcoholic solution of phenolphthalein, and to the third 4-5 drops of tincture of litmus. Then to each add just enough normal soda solution to give a permanent tint.

With the first glass (orange III. as indicator) the colour

change from red to yellow occurs when 10 cc. of soda have been run in. This point corresponds to the transformation of all the acid into monosodium phosphate,  $\text{NaH}_2\text{PO}_4$ , or, to be strictly accurate, to the addition of just a trace of alkali in excess of that needed to effect this combination.

With the glass containing phenolphthalein as indicator, the rose tint only appears after the addition of 20 cc. of soda, *i.e.* just twice the amount used in the first case; this corresponds to the conversion of the acid into disodium phosphate,  $\text{Na}_2\text{HPO}_4$ .

With litmus as indicator, a violet-red makes its appearance after the addition of 9 to 10 cc. of soda solution; with further addition of soda the violet-red becomes more and more blue, until when 20-22 cc. of alkali have been added the colour is frankly blue. In consequence of this lack of sharpness of definition in the colours produced, litmus should never be used as an indicator in the presence of phosphates.

The following table gives the reactions of phosphoric acid and the phosphates of alkali metals to the above-mentioned reagents.

	Orange III.	Litmus.	Phenolphthalein.
Free phosphoric acid $\text{H}_3\text{PO}_4$ . . .	Acid	Acid	Acid
Mono-metallic phosphate ( $\text{NaH}_2\text{PO}_4$ )	Neutral	Acid	Acid
Di-metallic        "       ( $\text{Na}_2\text{HPO}_4$ )	Alkaline	Alkaline	Neutral <sup>1</sup>
Tri-metallic       "       ( $\text{Na}_3\text{PO}_4$ ) .	Alkaline	Alkaline	Alkaline

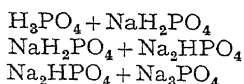
Of the phosphates of the alkaline earths, those corresponding to the above mono- and di-metallic types are both acid to these three indicators, while those corresponding to the tri-metallic type are neutral.

### Estimation of Phosphoric Acid and of Alkali Phosphates

68. Phosphoric acid and the phosphates of the alkali metals may occur mixed in various solutions; but on account of their different degrees of acidity and alkalinity and

<sup>1</sup> Phosphates of this type are in reality very faintly alkaline to phenolphthalein.

consequent reciprocal actions, the following combinations only can exist ; in the case, *e.g.*, of sodium.



Any other mixture made will be immediately transformed into one of these three types. It is easy to see by the combined use of the two indicators, orange III. and phenolphthalein, with which type of mixture one has to deal ; as is shown in the following table, wherein of course the Na may be replaced by K or  $\text{NH}_4$ , either wholly or in part.

	Orange III.	Phenolphthalein.
(1) $\text{H}_3\text{PO}_4 + \text{NaH}_2\text{PO}_4$	Acid (red)	Acid (colourless)
(2) $\text{NaH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$	Alkaline (yellow)	Acid (colourless)
(3) $\text{Na}_2\text{HPO}_4 + \text{Na}_3\text{PO}_4$	Alkaline (yellow)	Alkaline (rose)

69. (1) *If the mixture is acid to both indicators.* This may be due either to the presence of free phosphoric acid alone, or to a mixture of phosphoric acid and monosodium dihydrogen phosphate.

Take 10 cc. of the liquid, add a drop of orange III., then the soda solution (N/10 for example) until the red gradually changes to yellow ( $x$  cubic centimetres). The acid has thereby been changed into the monosodium compound.

Take another 10 cc. of the liquid and add a drop of phenolphthalein solution, then N/10 soda solution until a faint permanent pink is obtained ( $y$  cubic centimetres). The whole has then been changed to the disodium compound.

If now  $y = 2x$ , the original liquid contained phosphoric acid alone, for evidently in this case twice as much soda is needed to produce  $\text{Na}_2\text{HPO}_4$  as  $\text{NaH}_2\text{PO}_4$ .

But if  $y > 2x$ , the mixture present is  $\text{H}_3\text{PO}_4 + \text{NaH}_2\text{PO}_4$  ; in this case  $x$  represents the quantity of soda necessary to convert  $\text{H}_3\text{PO}_4$  to  $\text{NaH}_2\text{PO}_4$ . In other words,  $x$  gives the amount of phosphoric acid present, which is expressed, in grammes per litre, by  $x \times 0.098 \times 100$ .<sup>1</sup>

<sup>1</sup> It should be remembered that  $\text{H}_3\text{PO}_4 = 98$ .

$\text{NaH}_2\text{PO}_4 = 120$ .

$\text{Na}_2\text{HPO}_4 = 142$ .

$\text{Na}_3\text{PO}_4 = 164$ .

It is obvious that  $2x$  represents the amount of soda necessary to convert this phosphoric acid into  $\text{NaH}_2\text{PO}_4$ , and consequently  $(y - 2x)$  is the quantity of soda necessary to convert the  $\text{NaH}_2\text{PO}_4$  already present into  $\text{Na}_2\text{HPO}_4$ . In other words,  $(y - 2x)$  gives the quantity of monosodium phosphate, which is expressed in grammes per litre, by  $(y - 2x) \times 0.012 \times 100$ .

If the acidity of the liquid to orange III. disappears on the addition of a single drop of decinormal soda, monosodium phosphate alone is present which can be titrated with soda in the presence of phenolphthalein.

70. (2) *The solution is alkaline to orange III., but acid to phenolphthalein.* Here we have to deal with a mixture of mono- and di-sodium phosphates. To 10 cc. of the solution are added a drop of orange III. and then just sufficient N/10 sulphuric acid to produce a rose colour. Call this quantity of acid  $x$  cubic centimetres. The  $\text{Na}_2\text{HPO}_4$  originally present is changed to  $\text{NaH}_2\text{PO}_4$ , and  $x$  represents the quantity of soda corresponding to the disodium phosphate present, expressed in grammes per litre, by  $x \times 0.0142 \times 100$ .

Now to 10 cc. of the original liquid add a drop of phenolphthalein and sufficient N/10 soda to produce a pink colour ( $y$  cubic centimetres). It is easy to see that the  $\text{NaH}_2\text{PO}_4$  has been changed to  $\text{Na}_2\text{HPO}_4$ ; in other words,  $y$  measures the monosodium phosphate present. Expressed in grammes per litre this is  $y \times 0.012 \times 100$ .

71. (3) *The liquid is alkaline to both indicators.* Here we have to deal with tri-sodium phosphate alone, or a mixture of tri- and bi-sodium phosphates, or a mixture of tri-sodium phosphate with an excess of soda. To 10 cc. of the solution a drop of phenolphthalein is added. Decinormal sulphuric acid is next run in until the pink colour just disappears. Call this amount of acid  $x$  cubic centimetres. All the tri-sodium phosphate  $\text{Na}_3\text{PO}_4$  will then have been transformed to the disodium compound  $\text{Na}_2\text{HPO}_4$ .

If a further 10 cc. of the original liquid are taken, and after the addition of a drop of orange III., N/10 sulphuric acid is run in until the yellow tint is just replaced by a pink,

all the phosphates present will exist in the form of monosodium phosphate  $\text{NaH}_2\text{PO}_4$ . Call the amount of decinormal acid so run in  $y$  cubic centimetres. Now if  $y = 2x$ , the original solution contained tri-sodium phosphate alone, since it requires twice as much acid to transform it into  $\text{NaH}_2\text{PO}_4$  than into  $\text{Na}_2\text{HPO}_4$ .

But if  $y > 2x$ , the mixture contained  $\text{Na}_2\text{HPO}_4$  and  $\text{Na}_3\text{PO}_4$ . In this case  $x$  represents the amount of acid necessary to transform  $\text{Na}_3\text{PO}_4$  into  $\text{Na}_2\text{HPO}_4$ . In other words,  $x$  measures the tri-sodium phosphate. Expressed in grammes per litre, this quantity is  $x \times 0.164 \times 100$ .

Since  $2x$  represents the number of cubic centimetres of acid necessary to bring this same tri-sodium phosphate to the condition of the monosodium phosphate, the difference  $y - 2x$  gives the quantity of acid corresponding to the di-sodium phosphate. Expressed in grammes per litre, this is  $(y - 2x) \times 0.142 \times 100$ .

It is evident that if the alkalinity of this last mixture with phenolphthalein disappears upon the addition of a single drop of acid, we should be dealing with di-sodium phosphate only, which could easily be titrated in the presence of orange III.<sup>1</sup>

### Estimation of Phosphoric Acid

#### 72. Volumetric Determination by the Uranium Method.—

The principle of this method depends upon the formation of a uranium phosphate which is insoluble in acetic acid. The end point of the reaction is attained when a drop of the liquid gives a reddish-brown colour with potassium ferrocyanide, due to the formation of uranium ferrocyanide, and indicating the presence of an excess of uranium. This process is however tedious, and the titration may be carried out with cochineal as an indicator. This body changes from red to green in the presence of excess of uranium.

<sup>1</sup> When a mixture of tri-sodium phosphate and excess of soda is in question  $y > 2x$ . Here the difference  $y - x$  corresponds to the tri-sodium phosphate.

The colour change in cochineal is, however, difficult to observe accurately when one is dealing with complex substances such as urine. In such cases the two methods may be combined; cochineal being used to show when the operation is nearing completion, and potassium ferrocyanide to determine the exact end-point.

73. To 20 cubic centimetres of urine add 1 cc. of tincture of cochineal, and neutralize. If the urine is acid, add dilute ammonia, drop by drop, until a violet-red colour is produced; if on the other hand it is alkaline, add dilute nitric acid until the colour is a reddish-yellow. Next add 2 cc. of a 10 per cent solution of sodium acetate and 3 drops of glacial acetic acid. Bring the whole to boiling and run into the boiling mixture the standard uranium nitrate solution,<sup>1</sup> until a definite green colour is obtained. Remove a drop of the liquid on a glass rod and allow it to fall upon a minute crystal of potassium ferrocyanide; this will turn red or brown when the operation is complete.<sup>2</sup>

If no coloration occurs add more of the uranium solution, two drops at a time until a positive result is attained.

The solution of uranium nitrate is standardized by titrating it, as above, against 20 cubic centimetres of a solution of acid ammonium phosphate  $(\text{NH}_4)\text{H}_2\text{PO}_4$ , containing 3.24 grammes per litre. Each cubic centimetre of this corresponds to .002 gramme of phosphoric anhydride  $\text{P}_2\text{O}_5$ , and 1 litre corresponds to 2 grammes of  $\text{P}_2\text{O}_5$ .

When the uranium nitrate has been accurately standardized against the phosphate, and the necessary convection made in its phosphate value, the number of cubic centimetres obtained from titration of the urine multiplied by 2 will give the quantity of  $\text{P}_2\text{O}_5$  in the sample, expressed as grammes per litre.

74. *Gravimetric Estimation.*—The method is based upon the precipitation of phosphoric acid as ammonio-magnesium

<sup>1</sup> Standard uranium nitrate solution contains 40 grammes of uranium nitrate and 10 grammes of sodium acetate in 1 litre.

<sup>2</sup> The rapidity of development of the colour will vary with the size of the crystal used; hence it is desirable to employ these approximately the same size, say 1 c.mm.

phosphate ; this when calcined is converted into magnesium pyrophosphate, which is then weighed.

The cases presenting the greatest complexity are those where the phosphoric acid is combined not only with the alkali metals, but with calcium, magnesium, or iron, as in ashes of animal or vegetable origin, urine, etc. The precipitation of these metals by ammonia can be inhibited by ammonium citrate.

75. For the gravimetric estimation of phosphoric acid in urine the following method is used. To 50 cc. of urine are added 1 gramme of citric acid dissolved in a few cubic centimetres of water ; then 10 cc. of a 10 per cent solution of ammonium chloride, 3 or 4 centimetres of 10 per cent magnesium chloride ; finally ammonia is added drop by drop, with constant stirring, until a faint odour of ammonia is distinctly noticeable. A precipitate soon forms, and when no more comes down, a volume of ammonia equal to one-third of the total volume of liquid is added, the whole thoroughly mixed, covered and allowed to stand for twenty-four hours.<sup>1</sup>

The mixture is then decanted on to a filter, and the precipitate washed with water containing one-fifth of its volume of ammonia. Washing is continued until the filtrate, acidified with nitric acid, no longer gives a precipitate with silver nitrate. Finally, to wash all the precipitate on to the filter small quantities of the dilute ammonia are used (a few cubic centimetres at a time), the precipitate being removed from the sides of the beaker by a glass rod guarded by a piece of rubber tubing about 1-2 centimetres in length. The filter with its contents is dried in the air-bath at 100°. As much as possible of the dried precipitate is then detached by means of a camel-hair brush. The filter with its adherent residue is then incinerated in a porcelain crucible, and after cooling, the bulk of the precipitate is added to the contents of the crucible. This is again heated and slowly brought to redness, so as to transform the ammonio-magnesium phos-

<sup>1</sup> Two hours' standing is sufficient if the precipitation commences immediately ; under these conditions there is no risk of supersaturation with ammonio-magnesium phosphate.

phate  $\text{MgNH}_4(\text{PO}_4)$  into magnesium pyrophosphate  $\text{Mg}_2\text{P}_2\text{O}_7$ . The heating is stopped when the contents of the crucible become white or at least greyish.<sup>1</sup>

The phosphate so obtained contains a small amount of magnesium oxide derived from ammonio-magnesium urate, which is precipitated at the same time as the phosphate. For approximate estimations this may be disregarded, and the calcined precipitate directly weighed. For exact determinations, the residue is dissolved in 3-5 cubic centimetres of 50 per cent hydrochloric acid, and heated on the water-bath for an hour, so as to transform the pyrophosphate into orthophosphate. It is then diluted with a little water and filtered to remove any traces of carbon, if such are present; a crystal of citric acid is next added to the filtrate, which is then precipitated by addition of a slight excess of ammonia. Finally a volume of ammonia equal to one-third of that of the original liquid is added. The mixture is allowed to stand, and the precipitate collected on a filter is treated as described above. After cooling the pyrophosphate is weighed. The weight obtained multiplied by  $\cdot 6396 \times 20$  gives the amount of  $\text{P}_2\text{O}_5$  present in 1 litre of urine.

<sup>1</sup> If the precipitate remains black, it is allowed to cool, then moistened with a drop of nitric acid. This is then gently evaporated off, and the crucible and contents heated to redness. The precipitate will then be found to be completely white.



## CHAPTER III

### THE GLUCOSE GROUP AND HYDROLYSABLE SUGARS

#### Sugars

76. THE percentage composition of these bodies corresponds generally to the formula  $C_n(H_2O)_m$ , where  $n$  and  $m$  are variable quantities; on account of this they are often known by the unscientific name of "carbohydrates."

They include :

(1) The glucose group, or reducing sugars of the general formula  $C_nH_{2n}O_n$ , in which  $n$  varies from 3 to 6.

(2) The condensation products of the above, or "saccharides," to which must be added some natural derivatives; these latter are either substitution products such as methylglucose,  $C_nH_{2n-1}O_n \cdot CH_3$ , or hydrogen-addition products, such as the mannitols,  $C_nH_{2n+2}O_n$ .

The saccharides are formed by the condensation of 2, 3, 4 . . .  $n$  molecules of either the same or different sugars of the glucose type, with the elimination of 1, 2, 3 . . .  $(n-1)$  molecules of water; from this constitution they have received the names of disaccharides, trisaccharides, etc., and generally speaking for the higher members, polysaccharides.

The simpler members of the group are freely soluble in water, crystallizable, and have a sweet taste. According to the mode of linkage of the molecules which form them they may or may not have reducing properties. Upon hydrolysis they are split up into two or more molecules of the simpler sugars (hexoses) (see Table, p. 59); on this account they are termed hydrolysable sugars; while according to

their power of reducing Fehling's solution, or otherwise, they are divided into two groups—the reducing and the non-reducing sugars.

The more complex polysaccharides are in general either insoluble or only slightly soluble in cold water; they crystallize with difficulty or are even amorphous. From a biological standpoint they may be divided into nutritive polysaccharides and reserve polysaccharides.

In addition to the above-mentioned bodies, which all belong to the fatty series, there may be mentioned in this connection the inosite group, or, as they are sometimes called, the “sugars of the aromatic series.”

As regards the glucosides, these may be considered as compound saccharides in which one molecule of a sugar of the glucose group or of a hydrolysable sugar is linked to a different type of molecule, such as a phenol or an aromatic alcohol.

### Reactions of the Reducing Sugars

#### Action of Alkalies

77. Upon boiling a solution of glucose or other reducing sugar with a solution of potash or soda, or with lime, the liquid becomes more or less yellow owing to the production of caramel.

This reaction still occurs, though with less intensity, if instead of the caustic alkalies themselves alkaline salts (*e.g.* carbonates) are employed.

### Reducing Properties

#### Preparation of Alkali-Copper Mixture

78. On adding a solution of caustic potash to one of copper sulphate, a blue precipitate of copper hydrate is formed, which is insoluble in excess of potash. If, however, the solution contains certain organic substances such as glycerol, mannitol, glucose, tartrates, etc., which contain alcoholic groups, re-solution of the precipitate occurs. This observation forms the basis for the preparation of the

alkali-copper mixture first proposed by the French chemist Barreswill, but commonly known as "Fehling's solution."

A good reagent of this type is obtained by following the directions given by Pasteur. The two following solutions are prepared—

## I.

Copper sulphate	.	.	40 grammes
Water	.	.	300 cubic centimetres

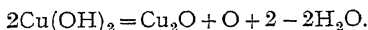
## II.

Caustic soda <sup>1</sup>	.	.	130 grammes
Neutral potassium tartrate	.	.	160 grammes
Water	.	.	700 cubic centimetres

The copper solution is added to the alkaline mixture with constant stirring and the whole boiled for a quarter of an hour. It is then allowed to settle; the supernatant liquid is carefully decanted, so as not to carry over any of the deposit, and then made up to 1 litre with distilled water.

The liquid so obtained must be preserved in the dark, and in course of time it deteriorates, so that it requires examination as follows before it is used for testing. One or two cubic centimetres are placed in a test-tube with 2 to 3 times their volume of water and well boiled; the fluid should remain perfectly clear. If it has deteriorated, a precipitate of cuprous oxide will be produced and it must be rejected.

79. *Method of Testing.*—Place a few cc. of the reagent in a test-tube, boil briskly, and add, drop by drop, a solution of some reducing sugar (*e.g.* glucose), when a red precipitate of cuprous oxide forms, the liquid at the same time being more or less decolorized. The cuprous oxide is formed by the reduction of cupric hydrate, a part of the oxygen being employed in the oxidation of the sugar.



. When there is excess of sugar, there is not only total

<sup>1</sup> The solid caustic soda may be replaced by 330 cc. of soda lye at 36° B. (p. xxxiii); then only 350 cc. of water are added.

precipitation of the copper, but the solution becomes yellow owing to the action of alkali upon the sugar (caramel).

If, however, only a small amount of sugar is present, and this in the presence of certain organic substances, the cuprous oxide instead of having a fine red colour and forming a dense precipitate, is orange, yellow, or even greenish, and precipitates with difficulty. This frequently occurs when urine is tested for the presence of sugar.

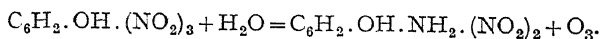
80. *Other Reducing Reactions.*—The reducing action of sugars containing an aldehyde or ketone group, when in an alkaline medium, occurs with other metals besides copper, such as mercury, bismuth, and silver, or with certain organic substances such as picric acid. This may be shown as follows.

To two or three cc. of a dilute solution of glucose are added a pastille of caustic potash and a small quantity of mercuric chloride. On heating, the original yellowish-white precipitate first becomes grey, then black.

81. By replacing the mercuric chloride of the preceding experiment with bismuth subnitrate, a white precipitate forms, which blackens on boiling. This test is very sensitive.

82. A test-tube is first thoroughly cleaned by boiling in it a mixture of nitric acid and lead oxide, and then carefully washed with distilled water. In it are placed 5 cc. of a 1 or 2 per cent solution of silver nitrate, and dilute ammonia is added, drop by drop, till the precipitate of silver oxide just dissolves. Two or three cc. of a dilute solution of glucose are next added, the mixture shaken, and carefully warmed. A brilliant mirror of metallic silver is deposited on the inside of the test-tube.

83. Certain organic bodies are also reduced by these sugars. To 4 or 5 cc. of picric acid solution add a slight excess of soda, then a drop of glucose solution, and boil; a blood-red colour appears, due to the reduction of picric to picramic acid.



### Furfurol Colour Reactions

84. If a reducing sugar is heated with a suitable concentration of acid, a furfurol compound is produced; this latter can combine with a phenol and form a compound whose colour depends upon the sugar and the phenol employed.

If orcinol and concentrated hydrochloric acid are used, the colour is violet-blue with the pentoses, orange-red with the methyl-pentoses and hexoses (G. Bertrand). With phloro-glucinol and concentrated hydrochloric acid the colour produced is red in all cases.

85. The procedure is as follows: Take 2 or 3 cc. of pure concentrated hydrochloric acid in a test-tube, add a few milligrammes of orcinol and of the sugar under examination; heat gently; in a few moments the characteristic colour appears, which, however, is often preceded by a pale yellow tint. On continuing to heat, the depth of colour increases and eventually a precipitate is formed. The heating must be very gentle ( $40^{\circ}$ - $50^{\circ}$ ) to avoid an unduly early formation of the precipitate, as the reaction is especially distinct in the earlier stages of the procedure, when the colouring matter is still in solution; moreover, too great heat may produce browning of the liquid, which will mask the coloration.

The reaction will also occur at the ordinary temperature, but then requires several hours for its completion. If the hydrochloric acid used contains traces of iron the colour given by the pentoses changes to green.

86. The following reaction (known as Séliwanoff's) enables us to distinguish between aldehydic and ketonic sugars; it depends upon the fact that if the hydrochloric acid used as above be *diluted with its own volume of water* the ketonic sugars alone give the reaction. It is mostly used for the detection of lævulose, and the phenol used is resorcinol; the colour obtained in this case is red.

We may note here that when pure reducing sugars are used to obtain these furfurol reactions, the colours so obtained

are also given by the substances whence they are experimentally derived. Thus xylane gives the violet-blue of the pentoses with orcinol and hydrochloric acid; starch and most glucosides give the orange-red of the hexoses under the same conditions; saccharose and inulin give Séliwanoff's reaction, etc.

### Preparation and Characters of Furfurol

87. Furfurol is produced by the distillation of bran or gum with hydrochloric or dilute sulphuric acid.

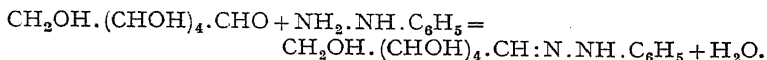
Five grammes of cherry-tree gum<sup>1</sup> (or gum arabic) are dissolved in 50 cc. of warm water and placed in a 250 cc. flask; 15 cc. of concentrated hydrochloric acid are next added together with a drop of oil to prevent frothing. Connect the flask to a condenser, distil slowly, and collect the distillate, which is an aqueous solution of furfurol. Observe the odour of the liquid and the following characteristic reactions.

88. *Orcinol Hydrochloric Acid Reaction*.—Proceed as in the case of examination for a pentose, but replacing the sugar by a single drop of the distillate (§ 87), so as to avoid dilution of the acid.

89. *Aniline Acetate Reaction*.—To a few cubic centimetres of furfurol solution are added a few drops of acetic acid and a drop of aniline; a vivid red colour is produced. Test papers soaked in aniline acetate and allowed to dry may also be used for this reaction.

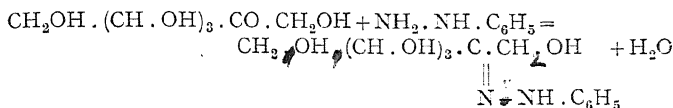
### Action of Phenylhydrazine on Sugars

90. *Preparation of Mannose-Hydrazone*.—Mannose gives with phenylhydrazine in the cold a characteristic compound which is only slightly soluble, and which serves for its recognition and separation. The compound results from the union of one molecule of each of the reacting substances with the elimination of one molecule of water.



<sup>1</sup> In place of the gum, a mixture of 10 grammes of bran with 50 cc. of water may be used.

In the case of ketonic sugars a similar reaction occurs, the changes being represented by

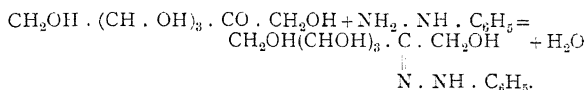


To prepare the mannose compound 180 grammes of mannose require 108 grammes of phenylhydrazine; in any given case the amount of mannose is roughly estimated and the calculated amount of phenylhydrazine (dissolved

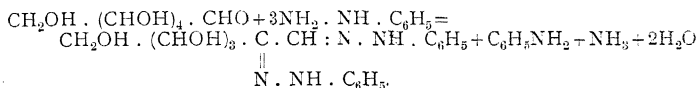
*Practical Biological Chemistry.*

## ERRATA

Page 49. The equation representing the action of phenylhydrazine upon ketonic sugars should read:



Page 50. The equation representing the formation of osazones should read:

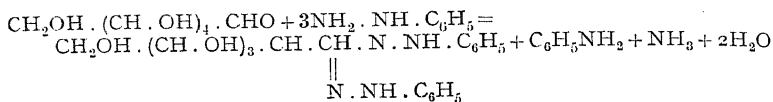


turbidity first appears and then a precipitate of mannose-hydrazone, which microscopically appears as crystalline spherules (Fig. 4). Its melting-point is 198°-199° (by Maquenne's block).

92. *Production of Sugar Osazones.*—All reducing sugars when treated with *excess* of phenylhydrazine acetate in the *warm*, form crystalline yellow compounds of slight solubility to which the name of osazones has been given. Here, two

<sup>1</sup> To prepare this, mix: Phenylhydrazine, 10 grammes; glacial acetic acid, 10 cubic centimetres; distilled water, 10 to 100 cubic centimetres.

molecules of the base combine with one molecule of the sugar; at the same time hydrogen is liberated and acts upon the excess of phenylhydrazine to produce aniline.



93. To prepare glucosazone, heat on the water-bath for an hour a mixture of 50 cc. of 1 per cent glucose solution with 10 cc. of the phenylhydrazine solution just described. Allow it to cool, and collect the crystalline deposit on a filter.



FIG. 5.—Glucosazone.

Wash it to the bottom of the filter with water and then several times with methyl alcohol, using in all about 20 cc. of the alcohol.<sup>1</sup> Dry the crystals by pressing them between layers of filter-paper, and they will thus be pure enough for the determination of their melting-point.

This procedure is manifestly only applicable to osazones which have but a slight solubility in methyl alcohol, such as glucosazone and galactosazone; those which are sufficiently soluble in water or organic solvents are dissolved in boiling water and recrystallized several times.

The osazones have very different melting-points (see p. 330),

<sup>1</sup> Their very slight solubility in methyl alcohol especially characterises glucosazone and galactosazone (G. Bertrand).



and very different and often characteristic crystalline forms. To examine them microscopically take a drop of the liquid containing them on a microscope slide, cover with a cover-

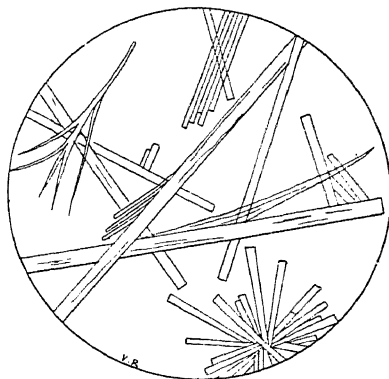


FIG. 6.—Galactosazone.

glass, and examine with a suitable objective. With a magnification of 500 diameters, glucosazone appears as yellow crystals in brush or fan-like groups of fine needles (Fig. 5).

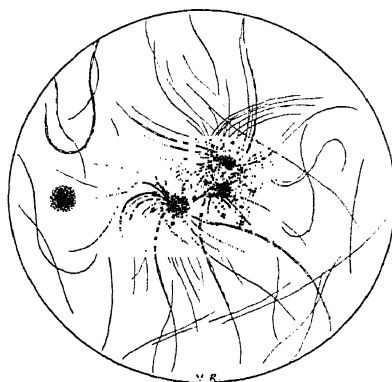


FIG. 7.—Arabinosazone.

Galactosazone under the same magnification appears as fine plates, lying either free or in groups of various forms and sizes (Fig. 6).

Arabinosazone forms very long fine filaments, which are

variously curved (Fig. 7); while xylosazone appears as beautiful long needles (Fig. 8), and maltosazone forms plates usually grouped in rosettes (Fig. 41).

94. *Investigation of Reducing Sugars in the Presence of Saccharose*.—If it is desired to test for a reducing sugar by the phenylhydrazine method when saccharose is also present, the phenylhydrazine solution; previously described in § 91,

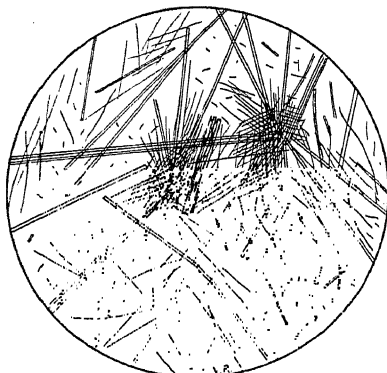


FIG. 8.—Xylosazone.

must not be used, as it contains an excess of acetic acid which would invert the saccharose; in its place the following reagent is employed.

Phenylhydrazine hydrochloride	. 3 grammes.
Sodium acetate	. . . . . 4.5 grammes.
Water	. . . to make up to 30 cubic centimetres.

Three cubic centimetres of this reagent are sufficient for .1 gramme of reducing sugar; the necessary heating should be carried out on the water-bath for an hour.

### Formation of Isomerides of Reducing Sugars by Alkalies

95. If a solution of one of the reducing sugars is left for some time with a little potash or soda, the sugar is partly converted into isomerides.

Glucose, for example, gives a mixture of lævulose and

mannose ; the conversion is not complete, a portion of the glucose remaining unaltered (Lobry de Bruyn and van Ekenstein).

This change is readily demonstrated in the case of glucose by taking 10 cc. of a 5 per cent solution of glucose in a test-tube and adding 1 cc. of a 10 per cent solution of potash. The tube is corked and left in the incubator at 35° for 24 hours. It is then cooled and 2 or 3 drops of acetic acid are added to neutralize the alkali and then 1 cc. of the solution of phenylhydrazine acetate (§ 91), the tube being well shaken. After a few seconds a turbidity appears and a precipitate of mannose-hydrazone (§ 90) is formed. The presence of lævulose can be demonstrated by taking a few drops of the liquid before the phenylhydrazine is added and testing by Séliwanoff's method (§ 86), when an intense red colour is obtained.

### Determination of Melting-Point

96. Many bodies undergo slight decomposition when heated almost to their melting-points; as a consequence the melting-point is depressed by these decomposition products. The decomposition is greater the longer the heating is continued, and the error of observation of the melting-point is correspondingly increased. To obtain accurate readings, therefore, it is necessary not to work at a temperature below that at which melting is practically instantaneous. This can be done by putting very small portions of the substance upon a block of metal heated to a known temperature, whereby the fragments of material and the metal block attain thermal equilibrium instantaneously (Maquenne's block).<sup>1</sup> To determine the melting-point of an osazone, for example, the sample is first dried and then finely powdered. The Maquenne block is heated so that the rise of temperature is fairly rapid (3-5° per minute). As each rise of 5° is noted a portion of the substance is put upon the block, and the temperature at which fusion is instantaneous is noted.

<sup>1</sup> For a description of Maquenne's block see p. 333.

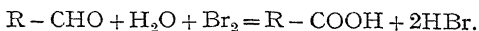
The block is then allowed to cool some degrees below this point. The temperature is again raised, but very slowly, so that the thermometer only shows a  $1^{\circ}$  rise in about three or four minutes. As each rise of  $1^{\circ}$  is noted, a fragment of the material is placed upon the block, so that the temperature at which instantaneous fusion occurs can be accurately observed.

Thus glucosazone melts between  $230-232^{\circ}$  when it is pure. Traces of impurities notably lower this melting-point, a fact which is of general application.

The method is unsuitable for bodies whose melting-point is lower than  $100-120^{\circ}$ , and especially for fatty substances. In these cases, the capillary tube method must be employed (see § 205).

### Acids derived from Sugars

97. *Monobasic Acids obtained by Oxidation with Bromine.*—Sugars which owe their reducing properties to an aldehydic group are easily oxidized by bromine in the presence of water, the  $-CHO$  group undergoing oxidation to  $-COOH$ .



Those on the other hand which possess a ketonic group do not undergo oxidation under these conditions; this property may be utilized to separate, for example, glucose and lævulose.

As an example of this transformation of an aldehyde-sugar to its corresponding monobasic acid, the oxidation of xylose may be taken. The xylonic acid thus produced has a very insoluble cadmium-bromine derivative, the formation of which is used in the detection of small quantities of "wood-sugar" or xylose.

98. *Preparation of Cadmium Xylonobromide.*—Place in a large test-tube 10 cc. of a 10 per cent solution of xylose, add .5 cc. of bromine, cork and shake gently from time to time to assist the solution of the bromine. After 48 hours the oxidation is finished, and excess of bromine is driven off by heating in a fume chamber (a suitable vessel should be at hand in which the tube can be placed if the evolution of

bromine is too rapid, so that it can be set down without spilling the contents). When this is complete, the liquid is turned into a little porcelain capsule and saturated with cadmium carbonate added by small portions at a time, the operation being assisted by careful heating. A small excess of the carbonate is added, and the whole is boiled for five minutes to decompose xylonic lactone which is also present. Filter while boiling; on cooling a precipitate of cadmium-xylonobromide ( $C_5H_9O_6Cd Br + H_2O$ ) is formed. Its formation is favoured by friction or by adding to the liquid half its volume of alcohol

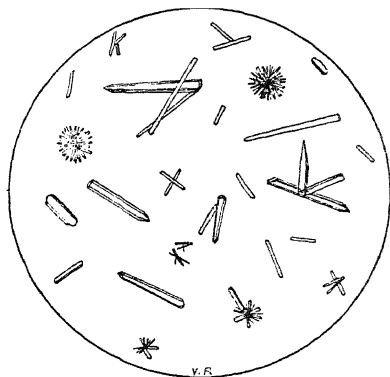


FIG. 9.—Cadmium Xylonobromide.

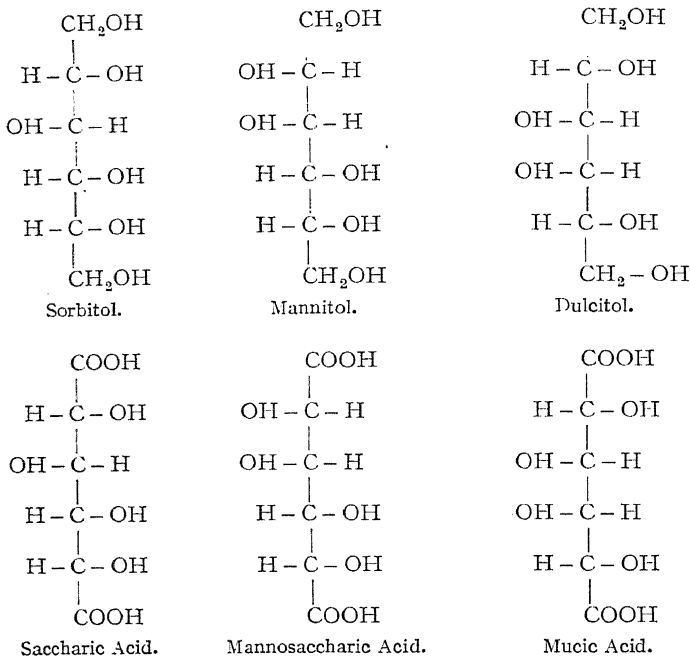
Under the microscope the precipitate shows as prismatic needles variously grouped, but often forming spheres (Fig. 9).

The salt so obtained does not give a constant melting-point; to obtain that, it is necessary to wash with 50 per cent alcohol and to dry again. If a portion is heated in a test-tube, it undergoes decomposition with a great deal of swelling up, similarly to mercury thiocyanate (G. Bertrand).

99. *Dibasic Acids formed by Oxidation of Sugars with Nitric Acid*.—When the aldehyde sugars are heated with dilute nitric acid they undergo a double oxidation, namely, in the aldehyde group and in the primary alcoholic group  $CH_2.OH$ , thereby giving a dibasic acid of the general formula



Glucose forms saccharic acid with a structural formula corresponding to that of sorbitol, mannose gives mannosaccharic acid corresponding to mannitol, and galactose is converted into mucic acid of analogous composition to dulcitol.



100. *Detection of Glucose by Formation of Saccharic Acid.*  
 —Heat on the water-bath, in an evaporating basin of 125 cc. capacity, 2 grammes of glucose and 10 cc. of nitric acid, S.G. 1.2,<sup>1</sup> until a brisk reaction occurs with evolution of reddish fumes (this should be done in a fume chamber); remove the flame, and when the reaction has quieted down, evaporate gently to a syrup to drive off excess of acid. Add 5-6 cc. of water, and saturate while warm with dry powdered potassium carbonate, turn the contents of the basin into a

<sup>1</sup> Obtained by mixing 2 parts of acid at 36° B. (see p. xxxii) with 1 part of water by weight.

beaker and add 3-4 cc. of glacial acetic acid. On stirring and cooling, a white crystalline precipitate of potassium hydrogen saccharate is thrown down. Microscopically it appears as sharp transparent needles often grouped in rosettes (Fig. 10).

101. *Detection of Galactose by the Formation of Mucic Acid.*—All bodies which furnish galactose upon hydrolysis produce mucic acid when oxidized. Thus lactose treated with nitric acid gives a mixture of saccharic acid (glucose) and mucic acid (galactose). The presence of the latter body is easy to establish since it is almost insoluble in water. The

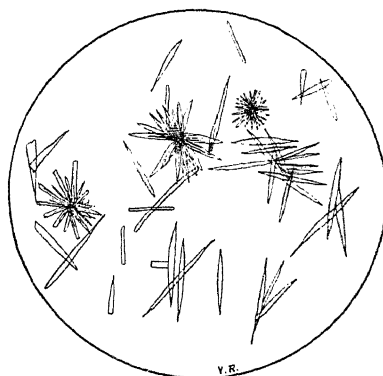


FIG. 10.—Acid Potassium Saccharate.

procedure is the same as for the oxidation of glucose, but when the oxidation is finished and the excess of nitric acid has been removed by evaporation,<sup>1</sup> the residue is taken up in 3-4 cc. of water and transferred to a beaker, the evaporating basin being washed out with distilled water, so that the total bulk amounts to about 10 cc. A white precipitate of mucic acid rapidly forms, which microscopically shows as small short prisms (Fig. 11).

The weight of mucic acid collected on a filter after twenty-four hours may be used as an approximate measure of the amount of galactose originally present; moreover,

<sup>1</sup> The evaporation is continued until the weight of the residue is twice that of the lactose originally taken, *i.e.* 4 grammes in this case.

1 gramme of lactose gives under these conditions about .33 gramme of mucic acid, when the latter is dried at  $110^{\circ}$ .

To verify that the precipitate is mucic acid, its solubility in ammonia should be tested; mucic acid should dissolve entirely. If, however, any calcium salt be present, it will

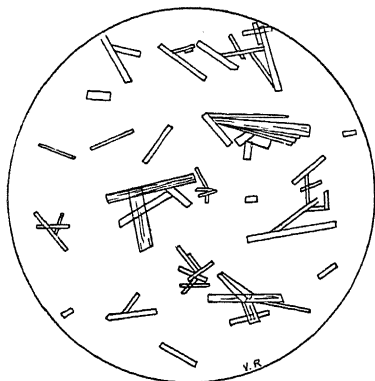


FIG. 11.—Mucic Acid.

also be precipitated (as oxalate), since the oxidation always produces a certain amount of oxalic acid. Calcium oxalate is only slightly soluble in dilute acids and is insoluble in ammonia.



# Principal Hydrolysable Sugars and Hexoses produced by Hydrolysis

Products of Hydrolysis.

Glucose. Galactose. Lævulose. Rhamnose.

DISACCHARIDES				
$C_{12}H_{22}O_{11}$				
Non-Reducing	{ Saccharose	1 mol.	—	1 mol.
	{ Trehalose	2 mols.	—	
Reducing	{ Maltose	2 mols.		
	{ Lactose.	1 mol.	1 mol.	
TRISACCHARIDE				
$C_{18}H_{32}O_{14}$				
Rhamninose . . .		1 mol.	—	2 mols.
TRISACCHARIDES				
$C_{18}H_{32}O_{16}$				
Raffinose . . . .	1 mol.	1 mol.	1 mol.	—
Melezitose . . . .	2 mols.	—	1 mol.	—
Gentianose . . . .	2 mols.	—	1 mol.	
Manninotriose . . .	1 mol.	2 mols.	—	
TETRASACCHARIDE				
$C_{24}H_{42}O_{21}$				
Stachyose . . . .	1 mol.	2 mols.	1 mol.	

## Inversion of Saccharose

102. If a few cubic centimetres of a dilute (1-2 per cent) solution of saccharose are boiled in a test-tube with an equal volume of alkali-copper solution, no reduction takes place. If, however, the saccharose has previously been boiled for a minute or two with a drop of hydrochloric acid, which is subsequently neutralized with 1 per cent soda solution, an abundant red precipitate is produced on boiling with Fehling's solution. Saccharose is decomposed by boiling with acids, into an equimolecular mixture of glucose and lævulose, both of which are reducing sugars.



### Colour Reaction of Lactose (Rubner)

103. If a solution of one of the reducing sugars is heated to boiling with the addition of lead acetate and ammonia, a colour is produced which varies from yellow to coppery red. By performing the test under standard conditions it can be so arranged that lactose alone gives a red, while the other sugars, especially glucose, give a yellow colour (Rubner). The reaction is so delicate that it may be used to distinguish lactose and glucose in solutions which are so dilute that other methods are unavailable ( $\cdot 5$  to 1 gramme per litre).

104. *Rubner's Reaction*.—To 10 cc. of a  $\cdot 1$  per cent lactose solution in a test-tube add about 1 gramme of crystalline lead acetate; warm gently to assist solution and add ammonia drop by drop, carefully shaking after each addition. At first the precipitate redissolves, but on further addition of ammonia (about 1 or 2 cc. are required in all) it persists sufficiently to render the liquid markedly turbid. The addition of ammonia is now stopped, as excess would inhibit the reaction, and the mixture is boiled for two or three minutes; a rose or orange colour appears, and after a few seconds a bright red precipitate is formed, the supernatant fluid being orange or rose coloured.

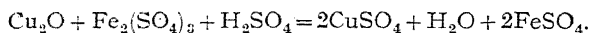
By treating a solution of glucose of the same concentration in a similar manner, a white or yellowish precipitate with a clear yellow supernatant fluid appears on standing.

With sugar solutions of higher concentration the amount of lead acetate and consequently of ammonia must be increased.

### Volumetric Estimation of Reducing Sugars (Bertrand's Method)

105. *Accurate Method* (Bertrand).—This consists in principle in boiling a known quantity of the sugar solution with excess of a standard solution of copper hydrate. The precipitated copper oxide is then titrated volumetrically according to the method devised by Mohr, which consists in

treating the precipitated cuprous oxide with an acid solution of ferric sulphate ; the oxide is dissolved and forms cupric sulphate, while a portion of the ferric sulphate is reduced to the ferrous condition,



The ferrous sulphate formed is titrated with potassium permanganate, and the amount of copper precipitated by the reducing sugar is calculated from the above equation.

The following solutions are necessary :

(A) Copper Solution.

Cupric sulphate . . . . .	40 grammes.
Dist. water, to make up to . . . .	1 litre.

(B) Alkaline Tartrate Solution.

Potassium-sodium tartrate . . . . .	200 grammes.
Caustic soda . . . . .	150 grammes. <sup>1</sup>
Dist. water, to make up to . . . . .	1 litre.

(C) Acid Ferric Sulphate Solution.

Ferric sulphate . . . . .	50 grammes.
Sulphuric acid . . . . .	200 grammes.
Dist. water, to make up to . . . . .	1 litre.

(D) Permanganate Solution.

Potassium permanganate . . . . .	5 grammes.
Dist. water, to make up to . . . . .	1

The ferric sulphate solution must not reduce permanganate. To ensure this, add a few drops of the permanganate to the ferric sulphate solution ; a faint colour change should appear even after a few drops. If this does not occur, add the permanganate drop by drop until the pink is *just* visible ; the iron solution is then ready for use.

106. To titrate the amount of reducing sugar in a given solution by this method, a conical flask of 125 to 150 cc. capacity is chosen, and 20 cc. of the sugar solution are intro-

<sup>1</sup> Instead of solid caustic soda, 375 cc. of soda solution (36° B.) may be used.

duced. These 20 cc. may contain up to 100 mgrs. sugar, but it is preferable that the sugar content should be a little less; the best results are obtained with quantities between 10 and 90 milligrammes.<sup>1</sup>

To the sugar solution add 20 cc. of the copper and 20 cc. of the alkaline tartrate solutions; heat to boiling, which is to be continued for exactly three minutes. It is important not to boil too rapidly in order to avoid undue concentration of the liquid. After three minutes' boiling the flask is removed, the precipitate of cuprous oxide is allowed to settle for a few seconds, and the supernatant liquid filtered through an asbestos filter. This liquid should by its colour clearly indicate the presence of an excess of copper; if this is not the case, too much sugar has been used and the experiment must be repeated.

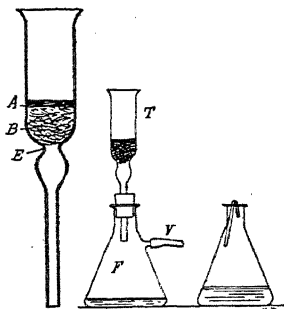


FIG. 12.—Asbestos Filter.

A. Filtering layer of fine asbestos.  
B. Coarse asbestos plug.  
E. Constriction to retain plug.

For the filtration, an asbestos-plugged tube of Soxhlet's pattern<sup>2</sup> (*T*, Fig. 12) is secured to a filter-flask (*F*) of 150 cc. capacity, which can be connected to a suitable filter-pump.

<sup>1</sup> If the necessary amount of sugar is contained in a less volume of liquid than 20 cc., the volume should be made up to this amount with distilled water, as the final volume must be constant.

<sup>2</sup> The tube containing the asbestos filter has a total length of 17 cm. The upper part is cylindrical, 6 cm. long and about 17 mm. in diameter. It is constricted at its lower end so as to retain the asbestos, and the constriction is a bulbous enlargement terminating in a tube which passes through the rubber stopper of the filter-flask.

An efficient filter is prepared by the following method. A specimen of asbestos is chosen which has rather firm, coarse fibres. This is made into a ball of the same diameter as the wide upper part of the tube and pushed down to the constriction (*E*) with slight pressure (*B*). Above it is put a thin layer of asbestos of much finer fibre (*A*), obtained by pounding ordinary asbestos with water. The finest portions are removed and set aside. The water is now poured into the tube and drawn through by the filter-pump. Finally, the finest fibres which were previously put aside are added, while exhaustion is in progress; they then form a superficial layer. It is this layer which forms the actual filter, and it should be at least 2-3 mm. thick, the other two layers simply acting as a support. The combined thickness

It is desirable to take over as little cuprous oxide as possible upon the asb stos filter, as an excess would form a compact layer and delay solution in the acid-iron mixture. When the supernatant liquid has been decanted from the cuprous oxide precipitate in the original flask, a little water is added, the precipitate again allowed to settle, and the washings decanted into the asbestos filter. The filter-flask is then exhausted, the filtrate thrown away, and the flask thoroughly cleaned; it is now ready for the second part of the operation, namely, the titration of the reduced cuprous oxide.

This is done by a very simple and rapid method: to the cuprous oxide remaining in the original boiling-flask is added enough of the acid-iron solution (5, 10, 20 cc.) to dissolve it, the whole being gently shaken to assist solution. On being so treated, the precipitate changes in colour from a red to a blue-black, and then gives a clear solution of a beautiful water-green colour. This solution is poured on to the asbestos filter (which has been readjusted to the filter-flask), and dissolves in its passage the small quantity of cuprous oxide deposited from the decanted liquid. If this does not dissolve sufficiently quickly, the surface of the filter is slightly moved with a stirring rod and the exhaustion stopped; if necessary, an additional known quantity of the acid-iron mixture is added.<sup>1</sup> Finally, when all the cuprous oxide has disappeared, the boiling-flask and the filter are washed through with distilled water, and the contents of the filter-flask titrated with permanganate solution. The colour change is extremely sharp, and is as well seen by artificial light as by daylight; the tint changes from green to rose with a single drop of permanganate in excess. Frequently almost all of the last drop of permanganate added is used up

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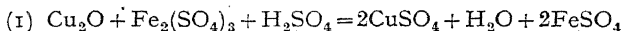
of the supporting layers is about 1 cm. It is important to see that each layer is regular in thickness and has a horizontal surface. With a little practice an asbestos filter which will serve for numerous filtrations (perhaps hundreds) can be prepared in a minute or two.

<sup>1</sup> After a considerable number of filtrations it may happen that the upper layer of the filter becomes blackened. In this case it is carefully removed, dried, burnt, and replaced.

in oxidizing the last traces of ferrous salt, and there only remains just enough permanganate to compensate the original green colour of the liquid, which then appears to be suddenly decolorized; on the addition of a single drop of permanganate an intense rose colour forms and persists.

The time required for these operations is from fifteen to twenty minutes.

As regards the calculations, it is clear from the equation



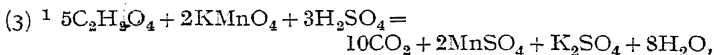
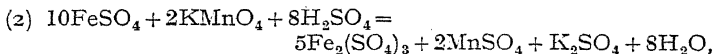
that two atoms of copper are precipitated as cuprous oxide corresponding to two molecules of ferrous sulphate, that is to say, to two atoms of ferrous iron to be oxidized by the permanganate.

It is only necessary then to determine previously the copper value of the permanganate solution (see § 107).

The accompanying tables (pp. 67-72) give the relations between different amounts of various reducing sugars and the amounts of copper precipitated by them (G. Bertrand).

107. It is better to use ammonium oxalate than ferrous sulphate for the titration of the permanganate solution, since it is easily obtained pure and is neither efflorescent nor hygroscopic; 250 milligrammes of ammonium oxalate are taken and put in a porcelain basin with 50 to 100 cc. of water, and 1 or 2 cc. of pure sulphuric acid, the whole is warmed to 60°-80°, and sufficient permanganate is run in to give a faint permanent pink (about 22 cc.).

From the following reaction<sup>1</sup>



it is seen that 1 molecule of oxalic acid, or what comes to

<sup>1</sup> The ammonia of the salt does not enter into the reaction but only the oxalic acid, which might be used *instead*. Oxalic acid, however, crystallizes with 2 molecules of H<sub>2</sub>O and is efflorescent. It is therefore not so convenient as its ammonium salt.

the same thing, 1 molecule of crystallized ammonium oxalate,  $C_2H_2O_4 \cdot 2NH_3 + H_2O$  (molecular weight 142.1), is equivalent to  $Fe_2$ , which again, from equation (1) above, is seen to be equivalent to  $Cu_2$ .

By multiplying the weight of oxalate by  $\frac{63.6 \times 2}{142.1}$ , or .895, we obtain the quantity of copper which corresponds to the amount of permanganate necessary to produce the rose colour. By dividing the number so obtained by the number of cubic centimetres of permanganate, the "titre" of the permanganate is obtained, in other words, the number of milligrammes of copper corresponding to each cubic centimetre of permanganate.

In round numbers, 1 litre of permanganate solution is equivalent to 10 grammes of copper; and indeed 10Fe (or 10Cu) correspond to  $2KMnO_4$ , or, taking the molecular weights, 1 gramme of  $KMnO_4$  corresponds to 2.01 grammes of copper.

The accompanying tables refer to weights of the various sugars in an anhydrous state.

For some sugars, of which the estimation is but rarely needed, it has not been thought necessary to give the figures for each milligramme; in such cases intervening figures can be interpolated without material loss of accuracy for most purposes.

Finally, by reason of the close resemblance between the tables for glucose and invert sugar, a special table for lævulose has not been given. Moreover, it is very rarely that one is called upon to titrate this sugar by itself, but should such a case arise the table for invert sugar will give sufficiently accurate readings.



TABLES.—Showing amounts of copper precipitated by different amounts of various reducing sugars, §§ 105-107.

## GLUCOSE

Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.
10	20.4	41	79.3	71	131.4
11	22.4	42	81.1	72	133.1
12	24.3	43	82.9	73	134.7
13	26.3	44	84.7	74	136.3
14	28.3	45		75	137.9
15	30.2	46		76	139.6
16	32.2	47	90.0	77	141.2
17	34.2	48	91.8	78	142.8
18	36.2	49	93.6	79	144.5
19	38.1	50	95.4	80	146.1
20	40.1	51	97.1	81	147.7
21	42.0	52	98.9	82	149.3
22	43.9	53	100.6	83	150.9
23	45.8	54	102.3	84	152.5
24	47.7	55	104.1	85	154.0
25	49.6	56	105.8	86	155.6
26	51.5	57	107.6	87	157.2
27	53.4	58	109.3	88	158.8
28	55.3	59	111.1	89	160.4
29	57.2	60	112.8	90	162.0
30	59.1	61	114.5	91	163.6
31	60.9	62	116.2	92	165.2
32	62.8	63	117.9	93	166.7
33	64.6	64	119.6	94	168.3
34	66.5	65	121.3	95	169.9
35	68.3	66	123.0	96	171.5
36	70.1	67	124.7	97	173.1
37	72.0	68	126.4	98	174.6
38	73.8	69	128.1	99	176.2
39	75.7	70	129.8	100	177.8
40	77.5				

## INVERT SUGAR

A .5 per cent solution obtained by hydrolysing 4.750 grammes of saccharose dissolved in 50 cubic centimetres of 2 per cent HCl and hydrolysed by heating to 100° for ten to fifteen minutes, cooling, neutralizing, and diluting to one litre.

Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.
10	20.6	41	79.5	71	130.8
11	22.6	42	81.2	72	132.4
12	24.6	43	83.0	73	134.0
13	26.5	44	84.8	74	135.6
14	28.5	45	86.5	75	137.2
15	30.5	46	88.3	76	138.9
16	32.5	47	90.1	77	140.5
17	34.5	48	91.9	78	142.1
18	36.4	49	93.6	79	143.7
19	38.4	50	95.4	80	145.3
20	40.4	51	97.1	81	146.9
21	42.3	52	98.8	82	148.5
22	44.2	53	100.6	83	150.0
23	46.1	54	102.3	84	151.6
24	48.0	55	104.0	85	153.2
25	49.8	56	105.7	86	154.8
26	51.7	57	107.4	87	156.4
27	53.6	58	109.2	88	157.9
28	55.5	59	110.9	89	159.5
29	57.4	60	112.6	90	161.1
30	59.3	61	114.3	91	162.6
31	61.1	62	115.9	92	164.2
32	63.0	63	117.6	93	165.7
33	64.8	64	119.2	94	167.3
34	66.7	65	120.9	95	168.8
35	68.5	66	122.6	96	170.3
36	70.3	67	124.2	97	171.9
37	72.2	68	125.9	98	173.4
38	74.0	69	127.5	99	175.0
39	75.9	70	129.2	100	176.5
40	77.7				

MANNOSE

Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.
10	20.7	60	113.3
20	40.5	70	130.2
30	59.5	80	146.9
40	78.0	90	163.3
50	95.9	100	179.4

GALACTOSE

Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.
10	19.3	40	73.9	70	125.0
11	21.2	41	75.6	71	126.6
12	23.0	42	77.4	72	128.3
13	24.9	43	79.1	73	130.0
14	26.7	44	80.8	74	131.5
15	28.6	45	82.5	75	133.1
16	30.5	46	84.3	76	134.8
17	32.3	47	86.0	77	136.4
18	34.2	48	87.7	78	138.0
19	36.0	49	89.5	79	139.7
20	37.9	50	91.2	80	141.3
21	39.7	51	92.9	81	142.7
22	41.6	52	94.6	82	144.6
23	43.4	53	96.3	83	146.2
24	45.2	54	98.0	84	147.8
25	47.0	55	99.7	85	149.4
26	48.9	56	101.5	86	151.1
27	50.7	57	103.2	87	152.7
28	52.5	58	104.9	88	154.3
29	54.4	59	106.6	89	156.6
30	56.2	60	108.3	90	157.6
31	58.0	61	110.0	91	159.2
32	59.7	62	111.6	92	160.8
33	61.5	63	113.3	93	162.4
34	63.3	64	115.0	94	164.0
35	65.0	65	116.6	95	165.6
36	66.8	66	118.3	96	167.2
37	68.6	67	120.0	97	168.8
38	70.4	68	121.7	98	170.4
39	72.1	69	123.3	99	172.0
				100	173.6

## SORBOSE

Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.
10	15.4	60	88.4
20	30.5	70	102.3
30	45.3	80	115.9
40	59.9	90	129.4
50	74.2	100	142.8

## RHAMNOSE

Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.
10	19.3	60	111.0
20	38.2	70	128.2
30	56.8	80	145.4
40	75.2	90	162.2
50	93.2	100	178.6

## ARABINOSE

Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.
10	21.2	60	119.3
20	41.9	70	137.5
30	62.0	80	155.3
40	81.5	90	172.7
50	100.6	100	189.8

## XYLOSE

Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.
10	20.1	60	113.2
20	39.6	70	130.6
30	58.7	80	147.6
40	77.3	90	164.2
50	95.4	100	180.5

## MALTOSE

The figures below refer to the anhydrous sugar.

Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.
10	11.2	41	45.2	71	77.6
11	12.3	42	46.3	72	78.6
12	13.4	43	47.4	73	79.7
13	14.5	44	48.5	74	80.8
14	15.6	45	49.5	75	81.8
15	16.7	46	50.6	76	82.9
16	17.8	47	51.7	77	84.0
17	18.9	48	52.8	78	85.1
18	20.0	49	53.9	79	86.1
19	21.1	50	55.0	80	87.2
20	22.2	51	56.1	81	88.3
21	23.3	52	57.1	82	89.4
22	24.4	53	58.2	83	90.4
23	25.5	54	59.3	84	91.5
24	26.6	55	60.3	85	92.6
25	27.7	56	61.4	86	93.7
26	28.9	57	62.5	87	94.8
27	30.0	58	63.5	88	95.8
28	31.1	59	64.6	89	96.9
29	32.2	60	65.7	90	98.0
30	33.3	61	66.8	91	99.0
31	34.4	62	67.9	92	100.1
32	35.5	63	68.9	93	101.1
33	36.5	64	70.0	94	102.2
34	37.6	65	71.1	95	103.2
35	38.7	66	72.2	96	104.2
36	39.8	67	73.3	97	105.2
37	40.9	68	74.3	98	106.3
38	41.9	69	75.4	99	107.4
39	43.0	70	76.5	100	108.4
40	44.1				

## LACTOSE

The figures below refer to the anhydrous sugar.

Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.	Sugar in milligr.	Copper in milligr.
10	14.4	41	56.7	71	95.4
11	15.8	42	58.0	72	96.6
12	17.2	43	59.3	73	97.9
13	18.6	44	60.6	74	99.1
14	20.0	45	61.9	75	100.4
15	21.4	46	63.3	76	101.7
16	22.8	47	64.6	77	102.9
17	24.2	48	65.9	78	104.2
18	25.6	49	67.2	79	105.4
19	27.9	50	68.5	80	106.7
20	28.4	51	69.8	81	107.9
21	29.8	52	71.1	82	109.2
22	31.1	53	72.4	83	110.4
23	32.5	54	73.7	84	111.7
24	33.9	55	74.9	85	112.9
25	35.2	56	76.2	86	114.1
26	36.6	57	77.5	87	115.4
27	38.0	58	78.8	88	116.6
28	39.4	59	80.1	89	117.9
29	40.7	60	81.4	90	119.1
30	42.1	61	82.7	91	120.3
31	43.4	62	83.9	92	121.6
32	44.8	63	85.2	93	122.8
33	46.1	64	86.5	94	124.0
34	47.4	65	87.7	95	125.2
35	48.7	66	89.0	96	126.5
36	50.1	67	90.3	97	127.7
37	51.4	68	91.6	98	128.9
38	52.7	69	92.8	99	130.2
39	54.1	70	94.1	100	131.4
40	55.4				

108. *Method by Decolorization.*—This method, which is very often employed, is simpler and looks more rapid than that just described. Owing to numerous circumstances which may influence decolorization (duration of the operation, partial reoxidation of the precipitate, etc.), it however furnishes less reliable results; greater precision can be obtained by repeating the titration, but in that case it takes

more time than Bertrand's method. To perform the titration a standard alkali-copper solution is used, a known volume of which is taken and decolorized by the sugar solution run in from a burette. The strength of the sugar solution should be about .5 per cent.<sup>1</sup>

109. Ten cubic centimetres of alkali-copper solution are accurately pipetted into a small flask of about 125 cc. capacity; 20 cc. of water are added, and the whole brought to boiling. The solution under examination is run into the boiling liquid, first of all in quantities of .5 cc. and then by single drops; the liquid is kept boiling and the flask agitated continuously. The operation is finished when the liquid is entirely decolorized, and it requires a certain amount of practice to determine this point precisely. Toward the end of the operation the flask is removed from the flame from time to time and the contents are allowed to settle for a moment; when the colour of the supernatant liquid is only very faintly coloured blue, the sugar solution must be run in only by tenths of a cc. at a time, or even by single drops, the boiling being continued for a minute after each addition. *If the supernatant liquid instead of being blue or colourless is yellow, it shows that there is an excess of sugar present.*

110. To titrate the alkali-copper solution, the same procedure is adopted, using a standard solution of glucose containing exactly .5 gramme of glucose per 100 cc. As it is difficult to obtain an absolutely pure glucose, and invert sugar has exactly the same reducing power, it is better to take saccharose and to invert it. For this purpose .475 gramme of saccharose is put into a graduated 100 cc. flask, with 50 cc. of water and 1 cc. of pure hydrochloric acid; the flask is then boiled on a water-bath for ten to fifteen minutes. After cooling, the acid is neutralized with a little soda or sodium carbonate solution, and the volume made up to 100 cc. The solution contains exactly 500 milligrammes of invert sugar, since 342 grammes of saccharose correspond to 360 grammes of invert sugar.

<sup>1</sup> If the sugar solution is more concentrated than .5 per cent, as determined by a preliminary rough estimation, it must be diluted until it approximates thereto.

The titre of alkali-copper solutions is measured by the number of milligrammes of glucose or of invert sugar necessary to exactly decolorize exactly 10 cc. of the reagent; it only undergoes slow deterioration when the solution is kept in the dark. The solution prepared according to Pasteur's formula (§ 78) has a titre of approximately 55.

When performing a titration, the calculation is made according to the following formula:  $C = \frac{T}{10v}$ , where C is the quantity of sugar in 100 cc. of the liquid, v the volume of the sugar solution necessary to decolorize 10 cc. of the Fehling's solution, and T the titre of the Fehling's solution.

If any other sugar than glucose or invert sugar is titrated by this method, the alkali-copper solution must be titrated for the sugar in question.

III. When it is merely necessary to make simple comparisons between different sugar solutions, such as to determine which has the maximum reducing power, etc., nothing else being sought than a simple approximation, a series of test-tubes may be taken, each containing 1 or 2 cc. of alkali-copper solution, with an equal volume of water. Each tube is boiled and the sugar solution run in drop by drop, the number of drops required to produce decolorization being counted. Naturally the richer the solution is in sugar the smaller the number of drops required.

### Polarimetry

III2. *Adjustment of the Apparatus.*—Before employing a polarimeter it is necessary to adjust the scale, and if necessary fix it for its zero point. We shall describe here the arrangement of Laurent's instrument.

Firstly, a yellow monochromatic light must be arranged in front of the instrument. This is easily obtained by putting a fused piece of an equimolecular mixture of sodium chloride and bisodium phosphate upon the nickel carrier of a Méker's burner. The scale is now turned from the zero point to the right or left, so as to divide the luminous disc into two



unequally illuminated halves. The eyepiece is adjusted so that the vertical line of separation in the luminous field is as sharp as possible. When this is done, the milled head is turned until the two halves of the illuminated field appear absolutely identical, so that it appears as a uniform luminous disc divided in two by a fine line: If the instrument is properly adjusted the zero of the scale and the zero of the vernier will exactly correspond. In practice it may happen, according to the amount of practice which the observer has had, that there is a slight difference to the right or left; this should not exceed two minutes. It is well to repeat this adjustment two or three times so as to determine the limit of approximation of the two zeros. Should the instrument not be sufficiently accurately adjusted—that is to say, if the error is greater than two minutes to the right or left—the two zeros are placed in exact coincidence, and the analyser is moved by means of the little screw (placed above the eyepiece) until the two halves of the illuminated field have exactly the same illumination. The adjustment of the instrument is now complete.

113. *Measurement of the Rotation produced by a given Solution.*—Carefully introduce the solution (by means of a pipette for example) into one of the tubes supplied with the polarimeter, which should be held slightly obliquely in order to avoid the inclusion of air-bubbles, until it forms a slight convex meniscus above the orifice of the tube, which is then held vertically. The round glass disc is now carefully slid over the extremity of the tube so as to remove the excess of liquid without the admission of air. The surplus liquid is wiped off, and the glass held in position by means of the bayonet catch.

The tube is now laid in the trough of the polarimeter, the cover is replaced, and the illuminated field examined through the eyepiece. If the two halves of the field appear equally illuminated, the liquid is devoid of rotatory power; if, however, one half is brighter than the other, it possesses optical activity.

Now the apparatus is so constructed that when the less

illuminated half disc is to the right the solution is dextro-rotatory; when the left half is the darker it is lævorotatory.

In order to measure the rotatory power, the two half discs must be brought to the same degree of brightness; this is done by turning the milled head and shifting the zero of the moving scale to the right if the solution is dextro-rotatory and to the left if it is lævorotatory, until the necessary equality of brightness of the two half discs is attained. The rotation obtained is read off by means of the attached lens, and is measured in degrees and minutes of the arc by the aid of the vernier. Several observations should always be made, by shifting the moving scale and readjusting it; the mean of the results obtained gives the final reading. It is useful, and in some cases necessary, at the moment of observation to note the temperature of the liquid under examination, as sometimes the rotatory power varies considerably according to the temperature (lævulose, invert sugar, etc.).

II4. *Calculation of the Specific Rotatory Power.*—The rotation observed is necessarily proportional to the specific rotatory power of the liquid examined, to the concentration of the solution, and to the length of the column of fluid traversed by the beam of light. The rotatory power is always expressed with a supposed concentration of 100 per cent, the deviation being supposed to be read through a column of fluid of 1 decimetre in length. Frequently longer tubes are used so as to magnify the deviation obtained. We have then

$$\alpha = \frac{[\alpha]_D \times p \times l}{100},$$

where  $\alpha$  represents the observed rotation,  $[\alpha]_D$  the specific rotatory power (for the sodium D line),  $l$  the length of the tube expressed in decimetres, and  $p$  the percentage strength of the solution.

From this it is easily deduced that

$$[\alpha]_D = \alpha \times \frac{1}{pl}$$

115. *Polarimetric Estimation of Glucose and Saccharose.*—

In the case of glucose, the percentage of sugar in a given solution is obtained by dividing the rotation (expressed in degrees and decimal fractions of a degree) obtained with a 20 cm. tube by 1.05. From the preceding formula it follows that

$$p = \frac{100a}{l \times [a]_D}$$

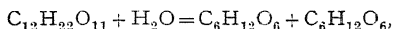
The specific rotatory power of glucose being  $52.5^\circ$ , we have

$$p = 2 \times 52.5$$

In the case of saccharose, either pure or mixed with glucose or other materials, the rotation  $a_1$  is determined in a 20 cm. tube (22 cm. if the volume has been augmented by 1/10 in decolorizing), the temperature of the solution being accurately noted.

Next 25 cc. of the liquid (supposed to be neutral) are taken and "inverted" by adding .5 cc. of pure hydrochloric acid and boiling for three or four minutes; the mixture is cooled, made up again to exactly 25 cc. and the rotation  $a_2$  observed, care being taken that the observation is made when the liquid is at the same temperature as before. The difference  $a_1 - a_2$  between the two observed rotations is solely due to the conversion of the saccharose into an equimolecular mixture of glucose and lævulose. Now the variation observed under these conditions with a 1 per cent solution of saccharose is at a temperature of  $0^\circ$ ,  $1.8445^\circ$  (in a 20 cm. tube), and at a temperature of  $t^\circ$  is  $(1.8445 - .0056t)^\circ$ .<sup>1</sup>

<sup>1</sup> Saccharose is hydrolysed according to the following equation :



whence it is seen that 1 gramme of saccharose yields on hydrolysis .5263 gramme of glucose, and the same quantity of lævulose. The rotations in a 20 cm. tube (at  $0^\circ$ ) for a 1 per cent solution are these :

$$\frac{1 \times 2 \times 66.5}{100} = 1.33^\circ \text{ before hydrolysis.}$$

If the difference  $\alpha_1 - \alpha_2$  is divided by this factor calculated for the experimental temperature, the percentage of saccharose in the solution is given.

The presence of other substances does not interfere with the validity of the reading, even though they possess rotatory power, provided that it does not vary during the process of inversion of the sugar.

116. *Estimation of a Mixture of Saccharose and Invert Sugar.*—This is a problem with two unknowns, and consequently two equations are necessary for its solution. These are obtained either by determining the reducing powers  $R_1$  and  $R_2$  before and after hydrolysis, or by measuring their polarimetric rotations under the same circumstances.

In the first case, the reducing power  $R_1$  will correspond to the original amount of invert sugar present, and  $R_2$  to the sum of the original invert sugar *plus* that produced by inversion. The difference  $(R_1 - R_2)$  multiplied by  $\frac{342}{360} (= .95)$  will give the proportion of saccharose.

If the optical method be chosen, the amount of saccharose is first estimated after the change in rotatory power produced by hydrolysis, as has been explained above (§ 115). The figure so obtained will enable the deviation corresponding to the saccharose before hydrolysis to be calculated ( $\alpha_2$ ). The difference between the rotation  $\alpha_1$  before hydrolysis and  $\alpha_2$  will give the deviation corresponding to the invert sugar.

The method of estimation by reduction is applicable to

$$\begin{array}{l} \frac{.5263 \times 2 \times 52.5}{100} = +.5526 \\ \frac{.5263 \times 2 \times 101.38}{100} = -1.0671 \end{array} \quad \left| \begin{array}{l} \\ - .5145 \text{ after hydrolysis.} \end{array} \right.$$

The difference  $\alpha_1 - \alpha_2$  is 1.8445 at a temperature of  $0^\circ$ ; since the rotatory power of lævulose diminishes with the temperature by .56t, we are able to arrive at the formula given in test. Similarly for a solution of maltose, the difference observed in a 20 cm. tube before and after hydrolysis, multiplied by the coefficient 15.048, gives the percentage of maltose present in the solution.

cases where the sugars are accompanied by optically active substances but not possessed of reducing power ; the optical method is used in those cases where the impurities are devoid of optical activity but have reducing properties.

### Decolorization of Sugar Solutions

117. Liquids which are designed for polarimetric observations must either be perfectly clear and colourless or only faintly tinged.

Solutions which are too deeply tinged can often be decolorized by shaking with a little animal charcoal and subsequent filtration. The process is more effectual if the mixture is heated on the water-bath for a few seconds, or if kept at the ordinary temperature for some hours with frequent shaking.

To clarify turbid solutions, if filtration is insufficient, recourse may be had to shaking with a little infusorial earth.

In many cases it is necessary to make use of a genuine purification by means of some suitable reagent which is without action upon the body whose rotatory power is to be measured.

118. *Estimation of Sugar in Urine.*—For example, to estimate the amount of sugar in a specimen of urine, the latter is decolorized by taking 50 cc. of urine and adding exactly 5 cc. (*i.e.*  $\frac{1}{10}$  of its volume) of a solution of basic lead acetate,<sup>1</sup> the mixture being well shaken and then filtered. It is then examined in a tube of 11 cm. or 22 cm. (instead of 10 cm. or 20 cm.) to allow for the added volume of sub-acetate solution, otherwise an addition of  $\frac{1}{10}$  must be made to the figure obtained.

119. *Estimation of Sugar in Fruit Syrups.*—With fruit

<sup>1</sup> To prepare basic lead acetate solution : 400 grammes of neutral lead acetate are dissolved in 1 litre of water, by boiling in a large evaporating basin ; 300 grammes of litharge finely powdered are added in small quantities to the boiling liquid, the whole being stirred continuously. When solution is practically complete the mixture is allowed to cool, and filtered.

syrops, which generally contain a mixture of saccharose and invert sugar, 20 grammes of syrup are accurately weighed out. This is placed in a 100 cc. graduated flask and diluted with 60 cc. of water. Then basic lead acetate solution is added, drop by drop, until the maximum degree of precipitation is obtained (1 to 2 cc.), *care being taken to avoid an excess which would redissolve the precipitate*. The volume is made up to 100 cc. with water, the whole being thoroughly mixed and then filtered. Exactly 50 cc. are next pipetted off, and pure sulphuric acid is added, drop by drop, so that a precipitate is formed. A pinch of pure calcium carbonate is added to neutralize completely any excess of acid; the mixture is shaken several times, its volume made up to 100 cc., and then filtered. The sugar is then estimated either by the reduction method or polarimetrically, each 10 cc. of the liquid corresponding to 1 gramme of syrup.

120. *Estimation of Lactose in Milk*.—To estimate sugar by the reduction method in a mixture rich in protein substances, such as milk, the proteins must first be precipitated by mercuric salts. To 50 cc. of milk exactly 5 cc. (or 1/10) of a mercuric sulphate solution<sup>1</sup> are added. After thoroughly shaking, soda solution is added, drop by drop, until the liquid is neutral, then a drop of acetic acid to render the reaction very faintly acid. The volume is made up to 100 cc. and the whole filtered through a pleated filter-paper.

The filtrate is shaken with a few pinches of powdered zinc, so as to remove all the mercury in solution, as this metal, if present, would be precipitated at the same time as the cuprous oxide in the subsequent operations, and would vitiate the readings. The total disappearance of all traces of mercury can be assured by taking a drop of the liquid and placing it on a freshly polished piece of copper (cleaned with sand and water); it should produce no trace of a grey stain. If such should appear even after an appreciable time, the liquid must again be shaken with some

<sup>1</sup> The mercuric sulphate solution is prepared by dissolving 350 grammes of mercuric sulphate in a mixture of 120 cc. of  $H_2SO_4$  and 750 cc. of water, on the water-bath. The volume is finally made up to 1 litre.

more zinc dust. When all the mercury has been removed the liquid is filtered and titrated.

If the estimation is to be conducted by means of the polarimeter it is only necessary to coagulate the proteins by a mixture of picric and acetic acids (25 cc. of .1 per cent solution of picric acid with .25 per cent acetic acid for each 25 cc. of milk). The mixture is then shaken and filtered.

## CHAPTER IV

### MANNITOLS

#### Preparation of the Acetic Ester of Mannitol

121. THE mannitols are bodies which consist entirely of alcoholic radicles, and consequently they give the usual reactions of alcohols ; they thus combine with acids to form esters. The alcohol is capable of fixing as many monovalent acid residues as it contains alcoholic hydroxyl groups ; thus mannitol forms a hexacetic ester.

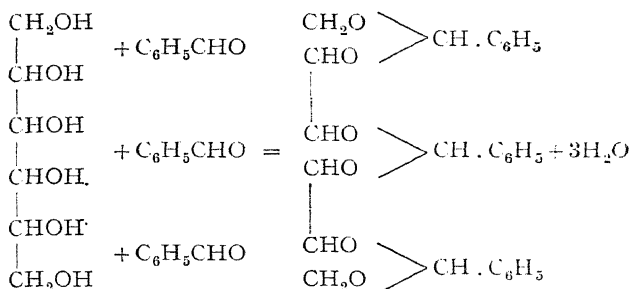
The hexacetic ester of mannitol can be obtained by heating, in a thoroughly dry test-tube, 1 gramme of mannitol with 4 or 5 grammes of acetic anhydride and a fragment of fused zinc chloride the size of a hemp seed. The zinc chloride is added to facilitate the reaction ; if it is not used it is difficult to obtain a complete acetylation. The heating must be done gradually, and with constant shaking of the tube ; as soon as the reaction commences the tube must be removed from the flame, as the heat generated by the change is sufficient for its completion. When the first violence of the reaction has calmed down, the tube is heated a second time to ensure that the operation is complete ; it is brought to boiling for half a minute and then allowed to cool. From 20 to 30 c.cm. of water are added to the contents of the tube, which is then well shaken, to dissolve excess of acetic anhydride and chloride of zinc. The acetic ester of mannitol precipitates as a syrup which soon becomes crystalline. The crystals are collected on a filter, washed with distilled water and allowed to dry, and the yield will be found to



equal the theoretical quantity which should be obtained. It can be purified by crystallization from warm alcohol at  $70^{\circ}$ , and has a melting-point of  $110^{\circ}$ . It must not be forgotten that the fumes of acetic anhydride are inflammable and highly irritating.

### Preparation of the Benzoic Acetal of Mannitol

122. The majority of polyatomic alcohols easily combine with aldehydes (benzoic, formic, etc.) to produce acetals. With benzoic aldehyde and mannitol the reaction is



Such a polyatomic alcohol as mannitol can fix half as many aldehyde residues, as it itself contains alcoholic hydroxyl groups.

The benzoic acetals in particular are easy to prepare; they are almost insoluble in water and serve for the identification and isolation of a number of polyatomic alcohols.

123. *Preparation of the Benzoic Acetal of Mannitol.*—Five grammes of manna are placed in a test-tube with a minimal quantity of water (say 1 cc.), and the whole warmed until it is dissolved to form a syrup. The syrup is run into a 50 cc. bottle with a wide mouth, and the test-tube rinsed out with 3 cc. of 50 per cent sulphuric acid (50 per cent by volume). This is added to the contents of the bottle, and the washing is repeated with the same volume of acid. To the mixture are added 3 cc. of benzoic aldehyde, the bottle is lightly corked <sup>1</sup> and thoroughly shaken.

<sup>1</sup> With a cork stopper.

A reaction occurs throughout the mass, though if the manna contains too many impurities the acetal is slow in forming, often requiring some hours or even until the following day. In such cases the shaking must be repeated several times.

After twenty-four hours the residue is washed by the aid of the filter-pump, receiving two or three washings with water to remove the mother-liquor in which the acetal was formed, and one with 60-70 per cent alcohol to remove the benzaldehyde. The product can be purified by recrystallization from alcohol.

To liberate the mannitol from its combination it is only necessary to boil its acetal with alcohol containing 2 per cent sulphuric acid until it completely dissolves. A current of steam is passed through the mixture to carry off the alcohol and the benzaldehyde which has been re-formed during the last reaction; finally, the sulphuric acid is removed by the addition of baryta, and mannitol alone is left in solution.

124. *Exhaustion and Drying with the Filter-Pump.*—Crystals, etc., are freed from superfluous liquid by being placed upon a round of filter-paper supported by a perforated porcelain plate which fits the funnel, which is connected through a filter-flask to the "filter-pump."

The porcelain plate is circular and the edges are oblique, so as to fit against the walls of a funnel whose walls incline at an angle of  $60^{\circ}$ . The funnel is fitted to a filter-flask of stout glass by a rubber cork, the filter-flask being connected to the filter-pump.

To commence operations a suitable round of paper is cut. The porcelain plate is placed with its larger surface downwards upon a sheet of filter-paper, and a disc is cut with a diameter about 1 cm. greater than that of the plate. A series of radial cuts are made round the edge of the paper up to the edge of the plate. When the round of paper is ready it is moistened with a few drops of water, the porcelain disc is placed in the funnel, and the paper placed upon its upper surface, so that the cut edges are applied to the sides of the funnel.

The apparatus having been arranged, the mixture to be treated is placed upon the filter-paper, carefully avoiding any shaking or disturbance of the funnel, and the filter-flask is connected to the pump. When no more liquid runs through, the residue is heaped up while gradually compressing it a little with some suitable instrument (*e.g.* a pestle, foot of a test-glass), and the exhaustion is continued until no more liquid passes through.

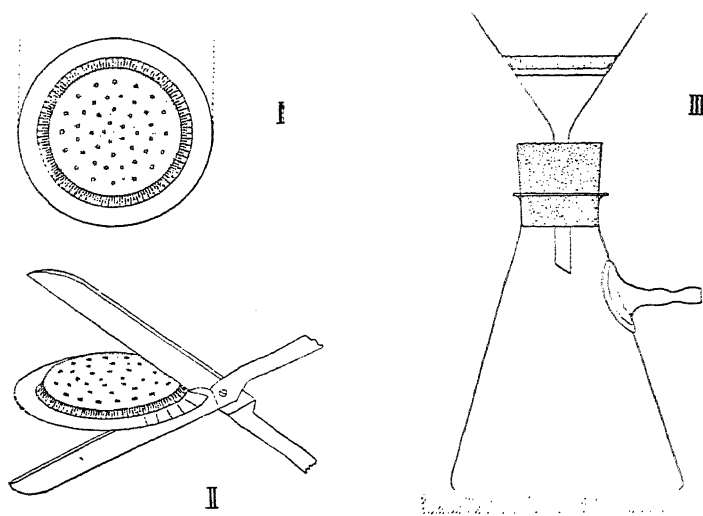


FIG. 13.—Method of arranging Funnel and Filter-Flask.

To effect complete purification the residue may be directly washed upon the filter with water or other suitable liquid; or better, it may be removed from the funnel (care being taken not to injure the paper) with a spatula, put into a mortar or capsule well mixed with the selected fluid, and the whole replaced on the paper in the funnel. After repeating these operations two or three times the washing may be considered complete.

### Inosite

125. Inosite, either free or in combination with phosphorus, is very widely distributed throughout the vegetable

kingdom. It is also found either free or combined in the muscles of various animals.

Chemically it is a hexatomic phenol, which on oxidation readily yields a quinone compound, rhodizonic acid, several of whose salts are coloured a bright red, and it is by means of them that the presence of inosite is demonstrated.

A fragment of inosite the size of a pin's head is put in a porcelain capsule and dissolved in 1 cc. of water with the addition of a single drop of Millon's reagent. It is evaporated to dryness on the water-bath and then dried over the flame at a temperature of 110°-120°. The resulting brownish residue is moistened with 3-4 cc. of glacial acetic acid, to which are added .5 cc. of water and a drop of a 10 per cent solution of strontium acetate. It is then evaporated again on the water-bath; the liquid soon acquires an eosin-red tint, and eventually a bright red deposit is left on the capsule.

126. Inosite can also be recognized by moistening a crystal with a drop of Gallois' reagent<sup>1</sup> and evaporating to dryness on the water-bath, when a red stain of rhodizionate of mercury will be left.

<sup>1</sup> Gallois' reagent is prepared by dissolving 1 gramme of yellow oxide of mercury in a mixture of 1 cc. of nitric acid and 10 cc. of water; the volume is then made up to 20 cc. with water.

## CHAPTER V

### THE HIGHER POLYSACCHARIDES

#### Microscopical Characters of Starches from Various Sources

127. STARCH, which forms the reserve food material of the majority of vegetables, occurs in the plant cells in the form of microscopic granules. To examine them, a particle of starch obtained by scraping a potato, haricot, pea, or grain of maize, etc., is mixed with a drop of water on a glass slide, the specimen covered with a cover-glass and examined microscopically with a suitable power. The granules are seen to be formed of concentric layers more or less united around a centre or hilum, which is the point of origin of the successive layers. The hilum is generally punctate, as in the case of potato or wheat starch; it is elongated or linear in starch from the bean and other leguminosæ; often when the grains are dried, small slits are seen radiating from the hilum, but they rarely attain any great length. The hilum is generally central in position; it is, however, eccentric in the case of potato and arrowroot.

The shape of the grain is very variable in different cases; ovoid and more or less irregular in potato and arrowroot, disc-like in wheat and barley, oval in the bean, and polyhedral in rice and maize (Figs. 14-18).

Generally the granules are isolated, but sometimes, as in the oat, several are agglomerated to form compound grains.

The size of the individual grains is very variable, as is seen from the following table:

Potato . . . . .	140-185 $\mu$
Arrowroot . . . . .	150-160
Broad Bean . . . . .	70-80
Haricot . . . . .	60-65
Pea . . . . .	45-55
Wheat . . . . .	45-55
Maize . . . . .	25-35
Oat (composite grains) . . . . .	35-45
„ (individual granules) . . . . .	6-8
Rice (individual granules) . . . . .	4-6

By reason of its mode of increase in size, the starch grain has up to a certain point a structure comparable with

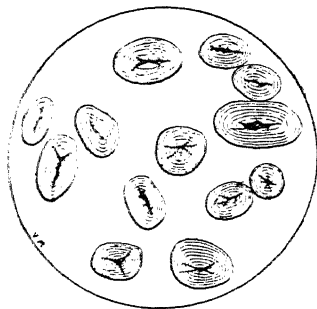


FIG. 14.—Haricot.

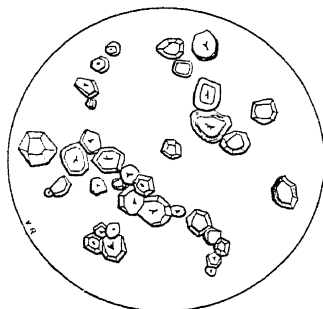


FIG. 15.—Maize.

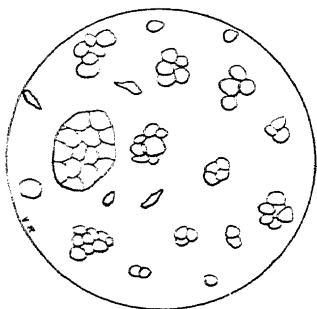


FIG. 16.—Oat.

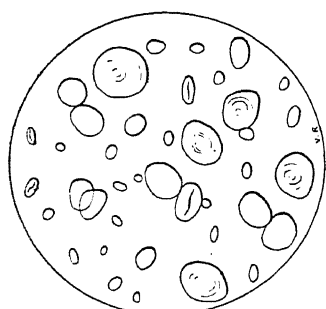


FIG. 17.—Wheat.

that of a spherical crystal, of which the hilum is the centre. If examined under crossed Nicol prisms there consequently appears, according to the shape of the grain, a more or less regular black cross, whose branches issue from the hilum.

By this means the hilum can be recognized even when it is not apparent with ordinary microscopical illumination.

128. *Action of Heat*.—The microscopical preparation is warmed above a small flame, but not to boiling. Water removed by evaporation is replaced if necessary by the addition of a small drop, placed at the side of the coverslip by a pipette, which then penetrates by capillary attraction between the slip and the slide. When the temperature reaches  $70^{\circ}$ - $80^{\circ}$ , according to the variety of starch, the grains swell markedly and are transformed into a paste. By

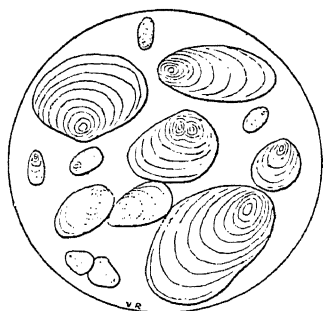


FIG. 18.—Potato.

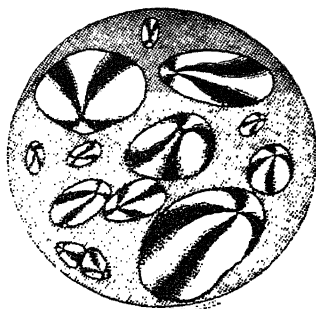


FIG. 19.—Potato (polarized light).

heating cautiously and watching the effects under the microscope it is often seen, early in the proceedings, that the various zones are better defined than in the unheated specimen.

129. *Action of Reagents. Potash*.—Alkalies cause a swelling of the starch grain and transform it into paste at the ordinary temperature. This reaction is well seen under the microscope by putting a drop of potash solution by the side of the cover-glass and allowing it to diffuse beneath.

The phenomenon of swelling can be observed and the effects of the alkali seen to be similar to those of heat.

130. *Action of Iodine*.—Iodine gives a very characteristic dark blue colour with starch.<sup>1</sup> To observe this under the

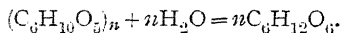
<sup>1</sup> In *Saponaria* (soapwort) and numerous other plants, there exists a glucoside (saponarin) in solution, which also gives a deep blue colour with iodine. The amyloid material found in certain pathological animal tissues gives a reddish-brown colour with iodine.

microscope put a drop of a solution of iodine in potassium iodide at the edge of the coverslip and allow it to diffuse beneath, or mount the starch in a very dilute iodine solution. The deep blue does not show well if there is too little iodine, but with a larger quantity the grains appear blue-black or sometimes brown. Upon warming the preparation the grains swell, the blue colour first becomes fainter and finally disappears. On cooling, the colour reappears, but the starch grains, of course, do not regain their original shape.

### Conversion of Starch to Sugar by Hydrochloric Acid

131. *Preparation of the Starch Paste.*—Weigh out 3 grammes of potato starch, add 20 to 25 cc. of water, and stir thoroughly. Heat to boiling in a beaker 125-130 cc. of water and pour the suspension of starch into it, stirring thoroughly meanwhile. The total volume should be made up to 150 cc.

132. *Conversion of Starch to Sugar.*—Three cubic centimetres of hydrochloric acid (at 22° B.) are added to the above paste, which is thoroughly stirred and brought to boiling. Previous to performing this hydrolysis, pipette off about 1 cc. of the liquid, put it in a test-tube with 10 cc. of water, and add 2 or 3 drops of iodine solution,<sup>1</sup> when the fine blue colour of iodide of starch will appear. While the boiling is in progress remove successive small quantities every three or four minutes and test it in the same manner. A series of tints will thus be obtained: blue, violet, reddish violet, red, mahogany, and chestnut. When the liquid gives no colour with iodine (after about 25 to 30 minutes), the boiling is stopped, and a further quantity of the solution tested with alkali-copper solution, which will be strongly reduced. The starch is transformed first into dextrin and then into glucose:



<sup>1</sup> To prepare the iodine solution: 1 gramme of iodine is ground up in a mortar with 2 grammes of potassium iodide and 2 or 3 cc. of water. This is gradually diluted with constant stirring, and the volume made up to 100 cc.



From the equation it is seen that 162 grammes of starch produce 180 grammes of glucose, or 9 parts of starch give 10 of reducing sugar. These figures may be made the basis of a method for estimating starch; to obtain quantitative results, however, it is necessary to employ prolonged heating in the autoclave.

### Glycogen

133. *Extraction of Glycogen from Molluscs.*—The hepatopancreas of the mussel, oyster, cockle, etc., is extremely rich in glycogen. In order to extract it, the body of the mollusc is first thoroughly broken up in a mincing machine; thirty grammes of the pulp so obtained are heated with an equal weight of strong potash solution (60 grammes of potash to 40 cc. of water) in a small porcelain dish, until all the tissue has disappeared (45 minutes to 1 hour). During the heating, water lost by evaporation is gradually replaced in proportion to the loss. Finally 50 cc. of water are added and the mixture filtered through an asbestos plug; the first portions of the filtrate are run through the filter a second time. To the filtrate is added half its volume of alcohol. A yellowish precipitate of glycogen falls, the supernatant liquid is run off on to a small unfolded filter, the precipitate is washed with dilute alcohol (alcohol 1 part, water 2 parts) and put on the filter. It is then well drained and dried between two layers of filter-paper.

134. If it is desired simply to study the properties of glycogen, the precipitate may be dissolved in a little warm water, and neutralized by acetic acid, when an opalescent solution is obtained.

A drop of iodine solution gives a mahogany-red colour with such a solution. If two or three drops of hydrochloric acid are added to a glycogen solution and the mixture boiled for a few minutes, it acquires the power of reducing alkali-copper solution, owing to the conversion of the glycogen to glucose.

135. *Centrifugalization.*—Since filtrations such as are mentioned above are tedious and difficult, it is preferable

to use the centrifuge for the collection and washing of specimens of glycogen. For this purpose the liquid to be centrifuged is put into one of the glass centrifuge tubes; this together with its metal case is placed in one pan of the balance, the corresponding centrifuge tube and case occupying the other. The two are adjusted to equal weight, either by placing a sufficient quantity of the liquid under treatment in the second tube or by putting into it a suitable quantity of water. The two tubes are then placed opposite to each other in the centrifuge, and the machine set in motion, which must be done gradually, care being taken to see that it is kept well oiled. When the centrifuging has proceeded long enough (from a few minutes to a quarter of an hour or even more, according to the nature of the liquid and the rate of revolution of the instrument), the machine is stopped. The precipitate should be clearly separated from the supernatant liquid and be sufficiently well compacted to allow of easy decantation. It can be washed by breaking it up with a glass rod and adding a little water; the stirring is continued with the addition of successive small amounts of water until sufficient of the latter has been added, care being taken to break up any lumps of precipitate. After having again adjusted the weight of the opposite centrifuge tube, the specimen is again centrifuged, and the deposit collected.

When there is a considerable bulk of liquid to be treated, so that it is too great for the series of tubes in the instrument, the turbid fluid may be added to the tubes after the first centrifugalization and decantation, without disturbing the original precipitates.

Generally speaking, centrifuging is more efficient than filtration, both as regards the time required and the separation obtained; for these reasons its use has displaced filtration in many operations.

136. *Detection of Glycogen in the Rabbit's Liver.*—The liver is removed immediately after the animal's death and cut up forthwith; 20 grammes are boiled with 20 cc. of the potash solution mentioned above. The subsequent pro-

cedures are the same as those for the extraction of glycogen from molluscs (§ 133).

Glycogen can be estimated like starch, by titrating the amount of glucose formed by its hydrolysis.

137. *Purification of Glycogen*.—When it is desired to purify glycogen obtained either from molluscs or from the rabbit's liver, it is dissolved in a minimal quantity of warm water (50°-60°); it is then cooled, and for every 100 cc. of solution, 10 grammes of crystallized potassium iodide and 5 cc. of 60 per cent solution of potash are added. After mixing, the glycogen is precipitated by the addition of 50 cc. of 96 per cent alcohol; the precipitate is drained, washed with a mixture of 50 cc. alcohol and 100 cc. of water with 5 cc. of 60 per cent potash solution and 10 grammes of potassium iodide, then drained completely. It is redissolved in water, neutralized by acetic acid, reprecipitated by alcohol and drained again. It is finally washed successively with 60 per cent, 75 per cent, 90 per cent, and 96 per cent alcohol; the white powder is dried at the ordinary temperature over sulphuric acid in a desiccator.

138. *Detection of Glycogen in Yeast*.—A twenty-four to forty-eight hours' old culture of yeast in beer-must contains cells which are rich in glycogen. If a little of the culture is examined under the microscope and a drop of iodine solution is placed at the side of the coverslip, so that it can diffuse beneath it, the contents of the cells are soon seen to acquire a more or less red-brown tint owing to the presence of glycogen.

### Hydrolysis of "Vegetable Ivory"

139. The kernel of an American palm (*Phytelephas macrocarpa*), used commercially under the name of "vegetable ivory" or corrozo, is composed almost entirely of manno-cellulose. Upon boiling with dilute acids mannose is formed, though the hydrolysis is less easily effected than is the case with starch and glycogen.

Five grammes of corrozo sawdust are boiled with 50 cc. of a 1 in 20 solution of hydrochloric acid (at 22° B.) in a

small flask fitted with a reflux condenser, the apparatus being placed in a fume cupboard. After at least an hour or two the boiling is stopped and the mannose separated as mannose-hydrazone (§§ 90-91). For this purpose, the contents of the flask are exactly neutralized by soda, the liquid is filtered and cooled, and 1 to 2 grammes of phenylhydrazine, previously dissolved in five times its weight of 20 per cent acetic acid, are added. On shaking, the mannose-hydrazone soon crystallizes out (§ 91).

### Soluble Gums

140. The soluble gums such as gum arabic and gum from the cherry tree mainly consist of higher polysaccharides (arabanes and galactanes). Their watery solutions are more or less thick; they give no characteristic colour with iodine, and are precipitated in large flocculent masses by alcohol or acetate of lead. On boiling with 5 per cent hydrochloric acid a mixture of reducing sugars is obtained, of which arabinose and galactose form a large proportion.

On oxidation by nitric acid the gums readily yield mucic acid, which is often mixed with a small quantity of calcium oxalate. It can be freed from this impurity by solution in ammonia (§ 101).

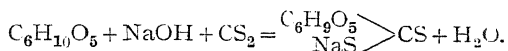
### Solution of Cellulose

141. The properties of cellulose can be studied either on filter-paper or on absorbent cotton-wool. Cellulose is insoluble in all the ordinary solvents, and resists for a long time any hydrolysis by boiling with dilute acids. Almost the only reagents capable of dissolving it are sulphuric acid, Schweitzer's reagent,<sup>1</sup> or the successive action of soda and carbon disulphide. The solution in Schweitzer's reagent has a syrupy consistence, and if it is added to excess of acidu-

<sup>1</sup> Schweitzer's reagent is prepared by allowing a current of air to bubble for several hours through a bottle containing ammonia and copper turnings. The operation is stopped when a strip of filter is almost immediately dissolved on being placed in a little of the blue liquid so obtained.

lated water, the cellulose is precipitated in white flakes, which obstinately retain traces of copper.

142. To obtain a solution of cellulose as a thiocarbonate, three grammes, *e.g.*, of absorbent cotton-wool are immersed in a 15 per cent solution of soda; excess of liquid is then expressed by means of a short thick stirring rod. The cotton-wool should retain about three times its own weight of soda solution. It is then placed in a stoppered flask with 1 cc. of carbon-disulphide and left for three hours at the ordinary temperature; sufficient water is then added to cover the mass and it is again left for some hours (overnight) until the solution is effected. Upon shaking, a homogeneous liquid is obtained, which can be diluted as required; the yellow colour is due to by-products (trithiocarbonates). The reaction is expressed by



### Colour Reactions of Cellulose

143. Cellulose when swollen by the action of certain reagents such as sulphuric acid or concentrated zinc chloride solution combines with iodine and gives a blue coloration like starch.

To obtain the reaction, soak a piece of filter-paper in a solution of iodine in potassium iodide, place it on a tile, and add cold slightly diluted sulphuric acid (2 parts acid at 66° B. and 1 part water). The portions impregnated with iodine become blue.

144. *Preparation of Chloriodide of Zinc.*—The sulphuric acid mentioned above can be replaced by concentrated phosphoric acid or chloride of zinc solution. For micro-chemical reactions a reagent known as “iodized chloride of zinc” is nearly always used; when treated with this reagent, cellulose immediately stains an intense blue.

To prepare the chloriodide take 25-30 cc. of a syrupy solution of chloride of zinc (at 45° B.); one or two grammes of crystallized potassium iodide and some iodine crystals

are next added. The mixture is heated over the naked flame with constant stirring so as gradually to concentrate the solution. From time to time a drop of the liquid is removed and allowed to fall upon a filter-paper; when a sufficient concentration has been obtained, it produces almost immediately an intense blue colour. Should the colour be slow in making its appearance the concentration must be continued a little further. Too great concentration must, however, be avoided, as the staining power is thereby lost, and it must be restored by the addition of a little water. The reagent acts more energetically when warm.

### Hydrolysis of Cellulose

145. Cellulose hydrolyses with difficulty under the action of boiling dilute acids; it requires previous solution in concentrated sulphuric acid. Under these conditions it is converted into glucose (Braconnot).

Mix in a small flask 25 grammes (or 14 cc.) of concentrated sulphuric acid and 8.5 cc. of water and allow to cool. Transfer the mixture to a beaker and add 5 grammes of filter-paper torn into small pieces; when a homogeneous mixture has been obtained by stirring, add half a litre of water and boil for an hour or two; the liquid acquires powerful reducing properties, but must of course be neutralized before testing with alkali-copper solution.

### Microchemical Reactions of Vegetable Tissues

146. *Differentiation of Woody and Cellulose Tissues.*—A thin section of a root or stem is mounted in a drop of chloriodide of zinc, covered and examined microscopically. The cellulose rapidly stains blue, while the woody elements are yellow.

A section may also be immersed in a little concentrated hydrochloric acid containing in solution a trace of orcinol, phloroglucinol, or vanillin (a fragment the size of a pin's head for 2 or 3 cc. of hydrochloric acid). There is an almost

instantaneous coloration of woody tissue, which gives a blue-violet with orcinol and a bright red with the two other reagents.

147. *Double Staining with Carmine and Malachite Green.*—The section is immersed for a few seconds in a solution of alkaline hypochlorite (eau de Javel); small bubbles of gas are evolved, the section is stirred about in the solution, then thoroughly washed in water and treated with alum-carmines,<sup>1</sup> with the addition of a *little* malachite green solution (10 per cent solution in 95 per cent alcohol). A suitable mixture is about 20 drops of carmine and 1 or 2 drops of malachite green solution. After about a minute the section is removed, washed with water in a watch-glass so as to remove excess of stain, and examined microscopically. The woody parts are stained green, the cellulose being red. If the differentiation is insufficient the section must be replaced in the stain and again washed and examined.

<sup>1</sup> Alum-carmines is prepared by boiling, for 10 to 20 minutes, 1 gramme of powdered carmine with 1.5 grammes of ordinary alum and 100 cc. of water in a small flask. The mixture is filtered when cool and a drop of phenol is added as a preservative.

## CHAPTER VI

### GLUCOSIDES

#### Preparation of Amygdalin

148. AMYGDALIN is a glucoside contained in the cotyledons of the bitter almond, apricot, peach, etc. It is usually obtained from bitter almonds. One hundred grammes of bitter almonds are first skinned by putting them into boiling water for a minute or two and then pressing them between the fingers. They are then chopped and added to 100 cc. of alcohol (90 per cent) in a flask, which is heated to boiling on the water-bath. This is done to destroy enzymes, which would partially decompose the glucoside. The flask is closed with a cork stopper through which passes a long tube to serve as a condenser, and kept gently boiling for ten minutes to dissolve the amygdalin. The alcohol is then decanted and the residue pressed to dryness in a cloth. The residue is then returned to the flask and extracted again by gently boiling with 50 cc. of alcohol (90 per cent) for ten minutes, when the alcohol is decanted and the residue expressed as before. The mixed alcoholic liquids, which are slightly turbid from the presence of oil dissolved during the heating, are thoroughly cooled, then passed through a folded filter previously moistened so as to retain the oil, collected in a flask of 500 cc. capacity, and then subjected to distillation *in vacuo*.

149. *Distillation "in vacuo."*—The flask<sup>1</sup> B containing

<sup>1</sup> A round-bottomed flask of even thickness throughout should be selected for distillation *in vacuo*. Flat-bottomed flasks should not be used.



the liquid to be distilled is closed with a perforated rubber stopper through which passes the large tube M with a lateral tubulure connected with the condenser R. All joints must be made with rubber bungs or tubes. At its upper extremity the tube M carries a bung through which passes a narrow glass tube with a finely-drawn-out end which reaches to within half a centimetre of the bottom of the flask. The tube is furnished with a piece of pressure tubing which can be closed by a short piece of glass rod, permitting a fine stream of air to enter the flask and facilitating

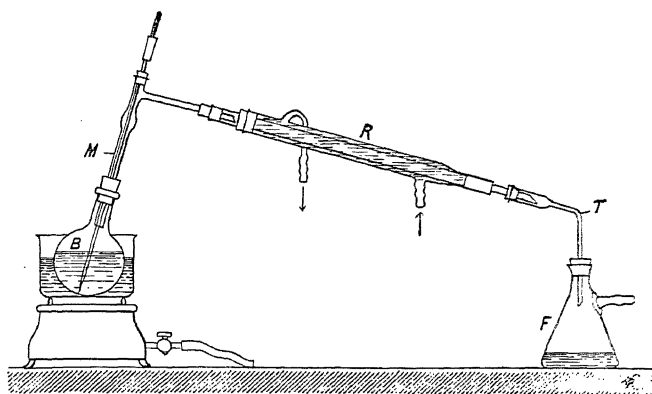


FIG. 20.—Distillation *in vacuo*.

boiling. The condenser is fitted to a bent collecting tube T at its lower end, and this passes through the stopper of a filter-flask of thick glass (F) and of about 300-400 cc. capacity.

When the apparatus is set up, the distilling-flask is arranged on a water-bath, and the tubulure of the filter-flask is connected to the pump by pressure tubing. The security of the joints must be tested by turning on the filter-pump and observing the manometer, which should not vary sensibly for several minutes. The distillation is started by exhausting as completely as possible, and gently warming the water-bath, care having been taken to set the water running in the jacket of the condenser.

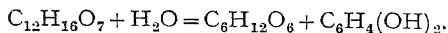
At the beginning a good deal of froth may form ; generally

this disappears rapidly and the distillation then proceeds in a normal and quiet manner. If, however, there are signs of the liquid frothing over into the condenser, a little air may be admitted through the fine tube, combined if necessary with cooling of the water-bath. Finally, if these measures prove inadequate a few drops of oil may be introduced with the aid of a pipette down the fine tube. When the distillation is finished, air is allowed slowly to enter the apparatus by gradually withdrawing the glass stopper, the filter-pump is disconnected and the distilling-flask taken down.

150. *Crystallization of Amygdalin*.—The syrup which remains in the flask is shaken up with 10 cc. of ether to remove traces of oil; the ethereal extract is decanted and the operation repeated two or three times. Crystallization often starts during these procedures, but without paying any attention to this partial crop of crystals, the whole contents of the flask should be turned into a porcelain dish, the flask itself washed out with a little warm alcohol, and the washings added to the contents of the dish. The whole is kept in a cool place for a few days, when the amygdalin will crystallize out. To purify it, wash the mass with a little ether, which is then drained off by means of the filter-pump (§ 124); finally, another washing with ether is given, and the residue drained and dried at the ordinary temperature.

### Decomposition of Arbutin

151. Arbutin decomposes by hydrolysis into a molecule of glucose and a molecule of hydroquinone :



The hydrolysis is effected by heating on the water-bath in a test-tube .5 gramme of the glucoside with 5 cc. of 5 per cent hydrochloric acid for an hour.

152. *Extraction of Hydroquinone*.—This diatomic phenol may be extracted from the products of hydrolysis of arbutin by means of ether. For this purpose the liquid is cooled and shaken up in a separating funnel with 10 cc. of ether.

On allowing to rest, the liquid separates into two layers; the lower is an acid solution of glucose, the upper an ethereal solution of hydroquinone. By carefully turning the stopcock of the funnel the sugar solution can be drawn off into a test-tube, and the ethereal solution is run into a glass beaker with vertical sides. To remove the last traces of hydroquinone the extraction with ether may be repeated; the ethereal extracts are of course mixed. The ether is evaporated off by placing the vessel containing it on a hot water-bath (the flame should be removed, as ether vapour is *exceedingly* inflammable), when the hydroquinone will crystallize in long white needles. It can be recognized by dissolving a little in 1 cc. of water and adding, drop by drop, a dilute solution of ferric chloride. A rose colour first appears, followed by a precipitate of quinhydrone in crystalline plates of a deep green colour with a metallic lustre. At the same time the characteristic odour of quinone is noticed. If excess of ferric chloride is used, quinone only is produced.

153. The glucose formed may be recognized as glucosazone. For this purpose .5 gramme of sodium acetate and a few drops of phenylhydrazine solution are added to 10 cc. of the acid liquid mentioned above. The whole is heated on the water-bath for about half an hour, when glucosazone will begin to crystallize out (see § 93).

## CHAPTER VII

### ACIDS OF THE FATTY SERIES

#### Characteristics of the Acids of the Acetic Series

154. THE saturated acids of the acetic series have the general formula  $C_nH_{2n}O_2$ . The lower members up to  $C_{10}$  are liquid and volatile in a current of steam; they are hence commonly known as "volatile acids."

The neutral solutions of their sodium salts give white precipitates on the addition of silver nitrate solution.

155. With ferric chloride the fatty acids give salts of a reddish-brown colour. These salts in the case of the three lowest members of the series are soluble in water, so that solutions of the formate, acetate, and propionate of sodium give a reddish coloration on the addition of ferric chloride. The tint changes to yellow on the addition of hydrochloric acid (distinction from thiocyanates). The ferric salts of butyric acid and its higher homologues form brown precipitates insoluble in water but soluble in ether (H. Agulhon).

156. The corresponding copper salts as regards their solubilities in water and ether resemble the ferric salts.

157. Heated with a little alcohol and concentrated sulphuric acid, the volatile acids or their sodium salts produce esters with characteristic odours. The formation of such esters forms a convenient method of testing for the lower fatty acids: control experiments should be made, using samples of acids of known purity.

### Formic Acid

158. A solution of formic acid, exactly neutralized by soda, gives a white precipitate with silver nitrate, which blackens on heating, owing to reduction and the consequent deposition of metallic silver.

159. Formic acid and the formates easily reduce a solution of chromic acid in nitric acid. By adding to 3 or 4 cc. of 5 per cent solution of potassium bichromate in nitric acid (36° B.) a drop of formic acid or a few crystals of a formate, a violet-blue colour is produced in the cold, owing to the formation of chromium nitrate. The other volatile acids do not give this reaction.

160. Upon adding a solution of neutral lead acetate to a solution of a formate, a white crystalline precipitate of lead formate is produced. This is soluble on heating, but re-crystallizes on cooling in small needles of great brilliancy.

### Acetic Acid

161. A solution of sodium acetate (which should not be too dilute) gives with silver nitrate a white crystalline precipitate, very slightly soluble in the cold but dissolving upon the application of heat. It easily re-crystallizes on cooling, forming white needles.

162. A small fragment of dry sodium acetate heated with half a cubic centimetre of alcohol and 10-20 drops of concentrated sulphuric acid gives a distinct odour of ethyl acetate. This is much more readily perceived if a little water is added (to suppress the odour of excess of alcohol).

163. A piece of sodium acetate the size of a pin's head, warmed with an equal quantity of powdered arsenious oxide, gives rise to the characteristic garlic-like odour of cacodyl oxide. The reaction is very sensitive, but by no means specific, as salts of the other volatile acids give products with similar odours.

## TESTS FOR THE VOLATILE FATTY ACIDS

Liquids more or less soluble in water, easily volatile in steam. Their alkali salts give precipitates with silver nitrate, which are only slightly soluble in water.

The silver salt rapidly blackens on heating; the original solution gives a <i>blue</i> coloration with a solution of potassium bichromate in nitric acid				FORMIC ACID.
To a solution of the sodium salt <sup>1</sup> is added an equal volume of ether, then, drop by drop, with continual shaking, a 2 per cent solution of copper sulphate.	The ether remains colourless.	To the original solution is added an equal volume of acetic ether, then, drop by drop, a 5 per cent solution of ferric chloride.	The ether remains colourless.  The ether is coloured yellow.	ACETIC ACID.
	The ether is coloured blue.			PROPIONIC ACID.
		To the original solution is added an equal volume of benzene, then, drop by drop, a 2 per cent solution of copper sulphate.	The benzene remains colourless.  The benzene is coloured blue.	BUTYRIC ACID.
				VALERIANIC ACID. CAPROIC ACID.

<sup>1</sup> Approximately a 1 per cent solution neutral to phenolphthalein.

### Butyric Acid

164. The odour of butyric acid is quite characteristic, namely, that of rancid butter. In dilute solutions, neutral to phenolphthalein, soluble butyrates give a blue precipitate with copper sulphate which is soluble in ether to a blue solution, but insoluble in benzene (H. Agulhon).

165. Upon heating a drop of butyric acid with half a cubic centimetre of alcohol and 10 drops of concentrated sulphuric acid, the characteristic odour of ethyl butyrate is given off (pine-apple odour).

### Oxalic Acid

166. The acid solution when completely neutralized by

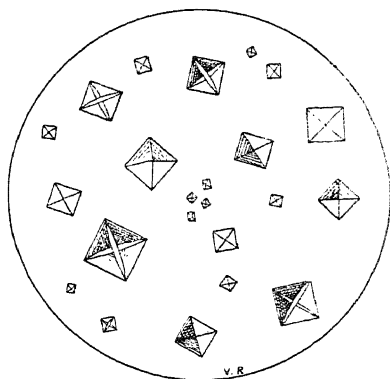


FIG. 21.—Calcium Oxalate.

ammonia gives a white precipitate on the addition of calcium chloride. This precipitate (calcium oxalate) is insoluble in neutral or alkaline solutions or in acetic acid; it is, however, soluble in mineral acids, when these are not too dilute. When calcined the precipitate of calcium oxalate leaves a residue of calcium carbonate.

If the amorphous precipitate of calcium oxalate is dissolved in just enough boiling 10 per cent hydrochloric acid to effect its solution, a clear liquid is obtained, which on

cooling deposits calcium oxalate as regular octahedral crystals; these can be examined under a suitable power of the microscope (Fig. 21).

167. *Detection of Oxalic Acid in Sorrel Leaves.*—Pound and express the juice from 200 grammes of sorrel leaves; boil the liquid to coagulate protein substances, filter, and precipitate the oxalic acid by calcium chloride in the presence of acetic acid.

### Succinic Acid

168. Succinic acid crystallizes from its ethereal solution in prisms which melt at  $185^{\circ}$ . The crystals when heated above their melting-point in a porcelain capsule are entirely volatilized with the formation of highly irritating white fumes.

169. If a crystal of succinic acid is placed in a watch-glass with a few drops of ammonia, the mixture gently heated to dryness to expel excess of ammonia and the residue dissolved in a little water, the solution will give a gelatinous ochre-coloured precipitate with ferric chloride (iron succinate).

### Lactic Acid

170. Lactic acid generally occurs as a thick syrupy liquid, which is freely soluble in water. It is easily extracted from its aqueous solutions by shaking with ether. It is not volatile with steam, and by this means may be distinguished from the volatile acids.<sup>1</sup>

171. By adding 1 cc. of 10 per cent sulphuric acid to 10 cc. of a solution of lactic acid or of a lactate, gradually heating the mixture to boiling and then adding, little by little, a 2 per cent solution of potassium permanganate, the

<sup>1</sup> A mixture of ferric chloride and phenol, known as Uffelmann's reagent, is often used for the detection of lactic acid. It is prepared by adding a few drops of a very dilute solution of ferric chloride to 10 cc. of a watery 1 per cent solution of phenol, when a blue-violet colour results. On adding a few drops of the acid solution to be tested, it will be found that mineral acids decolorize it completely, while lactic acid turns it yellow.

The reaction is not specific for lactic acid, since it is given by oxalic, tartaric, malic, and several other organic acids.



lactic acid is converted, almost quantitatively, to aldehyde. If a glass rod, moistened with a drop of ammoniacal silver nitrate solution, is held in the lumen of the tube a black deposit is formed upon it, due to the reduction of the silver salt to metallic silver by the aldehyde, which can at the same time be recognized by its characteristic odour (§ 504).

172. *Hopkins' Reaction*.—To a drop of lactic acid (or a few drops of an alcoholic solution of lactic acid) in a perfectly dry test-tube, are added 5 cc. of concentrated sulphuric acid and 3 drops of a saturated solution of copper sulphate. The mixture is thoroughly mixed and put in a bath of boiling water for five minutes; it is then rapidly cooled and 2 drops of a very dilute alcoholic solution (2 grammes per litre) of thiophene are added. On shaking and reheating, a cherry-red coloration is obtained.

This reaction appears to be given by all acids containing a primary or secondary alcoholic group; those containing only a tertiary alcoholic group do not give it. Thus with glycollic, malic, or gluconic acids, a rose colour is obtained, but much less intense than in the case of lactic acid. Glyceric acid gives a chestnut-red and tartaric acid a dirty violet.

Citric acid, which only contains a tertiary alcoholic group C.OH, does not give such a reaction, and the same is true of succinic acid, which does not contain any alcoholic group. Acids containing a phenol group (*e.g.* salicylic) similarly do not react in this way. It follows that the reaction may be applied for the recognition of lactic acid in animal tissues, when the acids mentioned above as giving Hopkins' test do not occur (P. Thomas).

173. *Lactate of Zinc*.—Heat to boiling 20 cc. of a 10 per cent solution of lactic acid and add, with constant stirring, small quantities of zinc carbonate ground into a paste with a little water. When saturation is nearly reached, add excess of zinc carbonate and leave on the water-bath, stirring the mixture frequently for half an hour. The mixture is filtered while still hot: on cooling, zinc lactate crystallizes out in transparent prisms, which should be examined under the microscope (Fig. 22).

Zinc lactate exists in two forms: an optically inactive variety containing three molecules of water of crystallization, and an active variety with two molecules of water, which rotates the plane of polarization either to the right or left. The water of crystallization is driven off by heating to  $110^{\circ}$ . These characters permit of the recognition of the type of

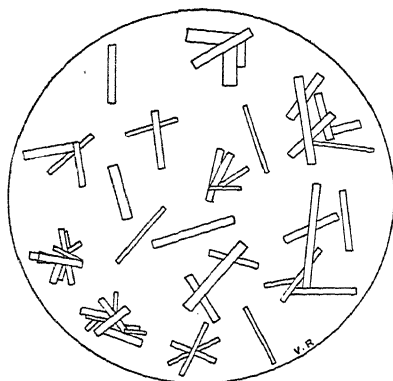


FIG. 22.—Zinc Lactate.

lactic acid produced in any particular fermentation; thus we have for the zinc lactates:

	Inactive.	Active.
Formula . . . .	$\text{Zn}(\text{C}_3\text{H}_5\text{O}_3)_2 + 3\text{H}_2\text{O}$	$\text{Zn}(\text{C}_3\text{H}_5\text{O}_3)_2 + 2\text{H}_2\text{O}$
Water . . . .	18.18 per cent	12.89 per cent
ZnO . . . .	27.27 per cent	29.03 per cent
Solubility at $15^{\circ}$ .	1.67 per cent	5.7 per cent
$[\alpha]_D$ . . . .	0	about $\pm 9^{\circ}$

### Malic Acid

174. Dry crystals of malic acid when heated in a test-tube first melt and then decompose, giving off water and white fumes of maleic acid. The latter condense upon the cooler parts of the tube, forming beautiful white needles.

175. A solution of malic acid, or of a malate of one of

the alkali metals, gives no precipitate with an excess of lime-water (added till the reaction is distinctly alkaline). The lime-water must be made by treating lime with hot water to saturation, since lime is less soluble in hot than in cold water. On boiling the mixture it remains clear ; this differentiates malic acid from tartaric acid, which precipitates with lime-water in the cold, and from citric acid, which precipitates with boiling lime-water.

### Tartaric Acid

176. A drop of a dilute solution of tartaric acid or of a

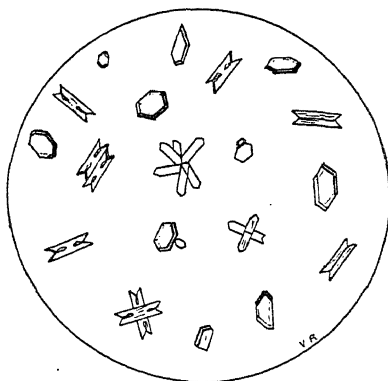


FIG. 23.—Potassium Bitartrate.

tartrate (1-2 grammes to a litre) placed in a test-tube with 2 drops of a 2 per cent solution of resorcinol and 3 cc. of concentrated sulphuric acid gives a liquid which on heating becomes first rose and then violet-red. The temperature must not be high enough to cause the acid to give off white fumes, since the colour then changes to brown and ultimately disappears, especially if the tartrate solution is too dilute.

A perceptible rose colour can be obtained with as small a quantity as .01 milligramme of tartaric acid. The other acids containing alcohol groups—lactic, malic, citric—either give no colour at all or only a yellow.

177. *Formation of Potassium Bitartrate.*—Two or three cubic centimetres of a solution of potash (1 in 10) are placed in a test-tube and neutralized by a solution of tartaric acid run in from a graduated pipette, a scrap of litmus-paper being used as indicator. The volume of acid necessary to effect neutralization is noted, and a further equal volume of acid added. If the solutions are not too dilute, a white crystalline precipitate of potassium hydrogen tartrate is rapidly formed, which should be examined under the microscope (Fig. 23). If the solution is too dilute, the formation of the precipitate can be hastened by the addition of alcohol.

178. To demonstrate the presence of tartaric acid in a solution (which should not be too dilute) a little potassium acetate and acetic acid are added. After a time, which varies with the strength of the solution used, small prismatic crystals of potassium hydrogen tartrate are formed. The formation of this precipitate is greatly facilitated by rubbing the sides of the test-tube with a glass rod, the crystals being first deposited at the sites of friction.

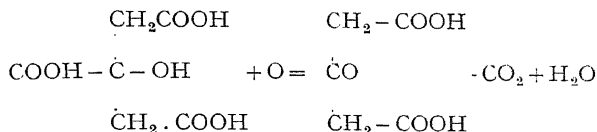
### Citric Acid

179. *Precipitation of Calcium Citrate.*—Ten cubic centimetres of lemon juice or of a 5 per cent solution of citric acid are carefully treated with strong soda solution until *very faintly* alkaline to litmus-paper; 5 cc. of a 10 per cent solution of calcium chloride are added. No change is observed; but on heating, an abundant white precipitate of tribasic citrate is produced.

180. *Denigès' Reaction.*—One cubic centimetre of the citric acid solution mentioned in § 179 is diluted to make a 1 in 50 solution. To 5 cc. of the diluted solution are added 3 cc. of mercuric sulphate in acid solution<sup>1</sup> (Denigès' reagent, which gives a white precipitate with ketones). The mixture remains clear, but if brought to boiling and a 2 per cent

<sup>1</sup> Denigès' solution is prepared by dissolving with the aid of heat 50 grammes of mercuric oxide (red or yellow) in a mixture of 200 cc. of pure sulphuric acid and 1 litre of distilled water.

solution of potassium permanganate is added drop by drop, the permanganate is first of all decolorized, but on cautious further addition the colour persists; finally the liquid becomes turbid and a white precipitate forms. This is due to a mercury compound of acetone-dicarboxylic acid, resulting from the oxidation of citric acid by the permanganate.



181. *Detection of Citric Acid in Milk.*—The above reaction serves for the detection of citric acid in milk, where it exists in small quantities (1-2 grammes per litre).

To 10 cc. of milk are added 2 cc. of a freshly prepared 5 per cent solution of metaphosphoric acid in order to precipitate proteins. The solutions are thoroughly mixed, and 3 cc. of Denigès' reagent are added; the whole is then well shaken and filtered. The filtrate is heated to boiling in a test-tube, and 2 per cent permanganate solution added drop by drop; a turbidity first appears and then a white precipitate. From 6 to 8 drops of permanganate are generally sufficient.

### Detection and Estimation of Acetic Acid in Vinegar

182. *Detection.*—Two hundred cubic centimetres of vinegar are distilled until three-fourths of the liquid have been collected. The distillate is exactly neutralized (using litmus-paper as indicator), and then evaporated to dryness on the water-bath. The residue is tested for acetic acid as above (§§ 161-163).

183. *Estimation.*—When acetic acid is mixed with other acids, as is the case with vinegar, advantage is taken of the fact that acetic acid is easily volatile in a current of steam. For this purpose 10 cc. of vinegar are put in the small distilling-flask (Fig. 24, D), which is connected with the condenser.

It is then connected to a one-litre boiling-flask (B) containing water to furnish the requisite steam. The steam bubbles through the vinegar and carries over the volatile acids. In order to avoid carrying over the liquid the tube which supplies the steam to the distilling-flask is furnished with two small bulbs (Fig. 24, *a*, *b*).

The heating of the small distilling-flask D must be carried out by a small flame, in such a manner that its contents are not increased in volume by condensation of the steam from B. If this precaution is neglected, the acetic acid in

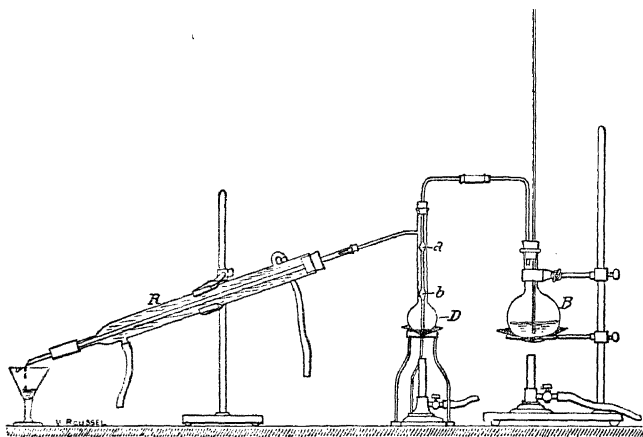


FIG. 24.—Apparatus for Estimation of Acetic Acid.

the distilling-flask becomes unduly diluted: and its removal by the current of steam is prolonged indefinitely. With a successful steam distillation the operation should be complete in from twenty to thirty minutes. The distillate is collected in a glass or beaker of 250 cc. capacity, and immediately titrated with normal or half-normal soda-solution, using phenolphthalein as indicator. The distillation is continued until the distillate is no longer acid.

The calculation is easy. Using normal soda-solution, each cubic centimetre of soda corresponds to 60 milligrammes of acetic acid, hence the acetic acid content of the vinegar expressed in grammes per litre is expressed by  $\cdot 060 \times 100 \times n$ .

or  $6n$ , where  $n$  represents the number of cc. of soda employed.

The total acidity of vinegar may be found (if it is not too highly coloured) by titrating a sample directly, having previously diluted it with two or three times its volume of water. Wine vinegar should have a higher total acidity than is accounted for by its acetic acid content, on account of the presence of succinic acid formed during alcoholic fermentation, and of acid potassium tartrate.

### Detection and Estimation of Succinic Acid in a Fermented Liquid

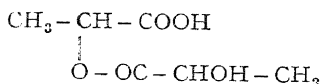
184. It is known that the amount of succinic acid produced during alcoholic fermentation varies from .3 to .7 per cent of the sugar fermented, and the following method devised by Pasteur may be employed for its detection and estimation.

The fermented liquid, carefully filtered, is put into a graduated flask; exactly 500 cc. are removed and evaporated *in vacuo* to about 10 cc., which are then transferred to a porcelain basin; the distilling-flask is washed out three times with a few cubic centimetres of warm water, and the washings are added to the contents of the basin. The flask must be thoroughly drained after each washing. The contents of the basin are now completely dried *in vacuo* over sulphuric acid in a desiccator. The syrupy residue is then treated with 10 cc. of a mixture of 1 part alcohol and 1.5 parts of pure ether. The liquid is decanted on to a folded filter, and the washing and filtering repeated six or seven times with the same liquid. Each time the washing is repeated the residue is well rubbed up with the alcohol-ether mixture, and from having a syrupy consistency becomes hard and friable. The final washings are collected in a flask, and the ether is then distilled off on the water-bath; the residue in the distilling-flask is then exactly neutralized with clear lime-water, phenolphthalein being chosen as the indicator. The mixture transferred to an evaporating basin is evaporated on the water-bath, and the

dry residue is treated with the alcohol-ether mixture which takes up any glycerol that is present. Calcium succinate remains behind as a crystalline residue, which is, however, contaminated with some extractive matters or uncrystallizable calcium salts; it is readily purified by digesting it (in the same evaporating basin) for twenty-four hours with alcohol (80 per cent). The alcohol dissolves the impurities and leaves a colourless crystalline deposit of calcium succinate, which may be collected, dried, and weighed upon a filter-paper. The weight of calcium succinate ( $C_4H_4O_4Ca + 3H_2O$ ) multiplied by 0.562 gives the amount of succinic acid contained in the quantity of liquid originally taken.

### Titration of a Solution of Lactic Acid

185. Aqueous solutions of lactic acid always contain a certain quantity of lactyl-lactic ester:



due to the interaction of two molecules of lactic acid.

The acidity of this body represents about half of that of the acids from which it was produced, and the quantitative relations between free acid and the ester vary with the concentration and the temperature. With increasing dilution and rise of temperature less of the ester is formed. When the free acid is completely saturated, equilibrium is disturbed, and a portion of the ester rapidly becomes converted into free acid even in the cold. Hence lactic acid requires special methods for its titration.

186. To determine the total acidity of a given solution of lactic acid, 10 cc. are taken, and normal soda very rapidly run in so as to give a faint pink with phenolphthalein; this, however, soon disappears. The quantity of soda  $x$  thus run in serves to neutralize the free acid and the acid group of the ester.

A known excess of soda (say 3 cc.) is then added, and the



mixture heated nearly to boiling for five minutes, to saponify the ester. After cooling, the excess of soda remaining is titrated with normal sulphuric acid. Since, however, the change from pink to colourless is difficult to define sharply, it is better to reverse the process, which is done as follows. After boiling and cooling exactly 5 cubic centimetres of normal acid (corresponding to the 5 cc. of normal soda originally used) are added. Then with constant stirring enough soda is run in, drop by drop, from the burette to give a pink colour. This quantity  $y$  corresponds to the lactic acid set free by saponification of the ester. The quantity of ester present, expressed in grammes per litre, is then  $y \times 162 \times 100$ , since the molecular weight of the ester is 162. The quantity of free acid is  $(x - y) \times 0.9 \times 100$ , the molecular weight of lactic acid being 90. The total quantity of lactic acid present, either free or combined as the ester, is  $(x + y) \times 0.9 \times 100$ .

In consequence of the instability of the colour change in the cold, the titration of the total acid is seldom exact.

### Detection and Estimation of Tartaric Acid in Wine

187. One of the most accurate methods of making this determination is that first suggested by Pasteur and perfected by Magnier de la Source. The wine is concentrated to a small volume and the acid potassium tartrate allowed to crystallize out. The crystals are collected and purified by washing. After drying they may be weighed or, better, titrated by means of standard alkali.

The procedure is as follows. Exactly 50 cubic centimetres of wine are taken in a porcelain dish, 1 cubic centimetre of a 10 per cent solution of potassium bromide is added, and the mixture evaporated to a clear syrup. It is then covered and allowed to stand for four or five days. At the end of this time crystallization of potassium bitartrate is complete. To the mixture are then added 10 cubic centimetres of a saturated solution of potassium bitartrate in 40 per cent alcohol. After mixing, the liquid is decanted on

to a filter, care being taken that as small a quantity as possible of the crystalline deposit passes over. The washing is repeated three times so as to remove colouring matter; the washings being decanted off as carefully as possible.

The filter is then washed with boiling water so as to dissolve any crystals which may have come away with the washings, and the hot filtrate is added to the main bulk of crystals so as to dissolve them. A drop of phenolphthalein is added, and standard baryta solution is run in to the liquid (which is kept warm) until a pink colour appears. The titration can also be made with decinormal soda, but in this case the liquid must be kept boiling (to drive off carbon dioxide, which may be produced from carbonates present in the soda) and litmus is used as the indicator.

Each cubic centimetre of decinormal soda corresponds to 0.0188 gramme of bitartrate.

## CHAPTER VIII

### PHENOLS AND AROMATIC ACIDS

#### Colour Reactions of the Phenols with Ferric Chloride

188. THE majority of phenols and of compounds containing a phenol group, when in neutral watery or alcoholic solution, give colour reactions with ferric chloride. The colours are generally shades of blue, green, violet, or red, and disappear on the addition of excess of the reagent. In order to see them as distinctly as possible, a very dilute solution of ferric chloride is added, drop by drop, to the liquid under examination. It is especially important to observe carefully the tint produced on the first addition of the reagent. Under these conditions the following colours are given by aqueous solutions.

Phenol (carbolic acid)	Blue.	Orcinol . . .	Violet blue.
Cresols . . . .	Blue.	Phloroglucinol	Violet blue.
Pyrocatechol . . .	Green.	Salicylic acid .	Deep violet.
Resorcinol . . . .	Violet blue.	Adrenaline . .	Green.
Hydroquinone . . .	{ Dark green ppt. of Quinhydrone.	Morphine. . .	Dark blue.
		Gallic acid . .	Blue black.

Guaiacol in aqueous solutions gives a more or less evanescent red due to the formation of tetraguaiacoquinone. In alcoholic solution it gives a blue, passing to green and ultimately to mahogany upon further addition of ferric chloride.

Pyrogallol in aqueous solution gives a yellow solution and a reddish-brown precipitate of purpurogallin. In

alcoholic solution it gives a fine blue, deepening and passing to black upon further addition of ferric chloride.

### Colour Reactions of the Phenols with Millon's Reagent

189. Ordinary phenol, the cresols, and most ortho- and para-derivatives (*e.g.* salicylic acid and tyrosine respectively) of phenols give a deep red colour with Millon's reagent, either in the cold or upon heating. This colour gradually deepens and gives place to a deep red precipitate.

Among the diatomic phenols, pyrocatechol, and its methyl derivative, guaiacol, give a deep red. Resorcinol and hydroquinone only give a yellow colour or precipitate, which does not correspond with the Millon reaction with ordinary phenol; orcinol gives a greenish precipitate.

Pyrogallol and phloroglucinol do not give Millon's reaction. It may be added that the naphthols  $\alpha$  and  $\beta$  give an orange colour followed by a brown precipitate; this phenomenon again does not correspond to the typical Millon reaction.

### Benzoic Acid

190. Benzoic acid forms white pearly spangles melting at  $121.5^{\circ}$ . It very easily sublimes, and is volatile in a current of steam. Though very soluble in boiling water, it needs 350 to 400 parts of water for its solution at the ordinary temperature.

To extract it from solutions, the solution is first acidified with sulphuric acid, then shaken with ether in a separating funnel (§ 152). The benzoic acid is easily and completely taken up by the ether, which it is only necessary to evaporate at a low temperature in order to obtain a crystalline residue containing the acid; benzoic acid may then be recognized by its solubility in boiling water, from which it crystallizes in needles on cooling, and by its melting-point.<sup>1</sup>

<sup>1</sup> The statement is frequently found in books that benzoic acid gives a colour reaction (the formation of aniline blue) when heated with a solution of fuchsin in aniline. Not only does benzoic acid not enter into the actual reaction (but merely serves to facilitate it), but the reaction itself is given by a variety of organic acids, such as acetic, succinic, cinnamic, and salicylic acids. The reaction when obtained is thus seen to be valueless as a test.

191. The following reaction, which is based upon the easy oxidation of benzoic to salicylic acid, may also be used.

To 5 cc. of a watery solution of benzoic acid, add 1 drop of glacial acetic acid, 5 drops of a 10 per cent solution of ferric chloride, and 5 drops of hydrogen peroxide (1 vol.). Gradually heat to boiling, and boil for 10-15 seconds. A reddish-violet colour is obtained, even if the solution only contains half a milligramme of benzoic acid.

### Salicylic Acid

192. Salicylic acid occurs as white crystals melting at 156°, and is as little soluble in water as benzoic acid. It dissolves in twelve and a half parts of boiling and 450 parts of cold water.

In watery solution it gives with ferric chloride a fine violet colour, due to the presence of its phenol group.

Upon heating some crystals of salicylic acid with one cubic centimetre of methyl alcohol and 10 drops of concentrated sulphuric acid, the characteristic odour of methyl salicylate (oil of wintergreen) is obtained.

### Formation of Salicylic Acid by Saponification of Oil of Wintergreen

193. This volatile oil contains salicylic acid in the form of methyl salicylate, which can be saponified by alcoholic potash in the following manner. In a test-tube put 1 cc. of oil of wintergreen, 1 pastille of potash and 2 cc. of absolute alcohol to act as solvent. Gradually warm up to boiling, and allow to boil for a few minutes. When the saponification is finished, the mixture is acidified with dilute hydrochloric acid (using a scrap of litmus-paper as indicator), boiled for a minute or two to drive off the alcohol, allowed to cool and extracted with ether (§ 152).

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Similarly with the test depending on nitration by nitric acid and subsequent reduction by ammonium sulphide to form a red amino compound. This is also given by numerous bodies of the aromatic series.

The ethereal extract is evaporated in a vertical-sided glass capsule, over hot water, when salicylic acid crystallizes out in white needles.

### The Tannins

194. The tannins form a group which it is difficult to define exactly. They are soluble in water, giving a yellow, brown, or red solution. They give a voluminous flocculent precipitate with watery solutions of gelatine and of alkaloids (sulphates of quinine, strychnine, caffeine, etc.).

They generally contain several phenol groups and hence give the usual reactions of the phenols; many contain one or more carboxyl groups, and many have the characters of glucosides.

In alkaline solutions they absorb oxygen from the air, yielding darkly coloured products. The majority of them, when in dilute watery solutions, give under the action of oxidases (laccase, tyrosinase, etc., § 431) dark coloured oxidation products, which are generally red or brown.

195. The tannin in the oak-gall gives with very dilute ferric salts an intense blue-black colour and precipitate; with ferrous salts, no precipitate is formed, but on exposure to air the mixture gradually first becomes brown and then deep blue-black. Other metallic salts give differently coloured precipitates with this tannin, thus:

Lead salts	.	.	.	.	.	.	.	White ppt.
Copper salts	.	.	.	.	.	.	.	Brown ppt.
Mercuric salts	.	.	.	.	.	.	.	Orange ppt.

Among the various tannins occurring in the vegetable kingdom those which are derived from gallic acid generally give a blue-black colour with ferric chloride (ink), while those derived from pyrocatechol (coffee, cinchona) give a green.

### Estimation of Tannin in Tanning Materials

196. From a practical point of view the most important property of tannin is its power of acting upon hides so as to produce leather. It is upon this property then that an

estimation of the amount of tannin present in any tanning material is based. For this purpose samples of powdered hide are used ; the tanning solution is allowed to act upon these ; the difference in the weights of the dissolved materials in the solution before and after treatment is held to give the weight of tannin present.

To this end, such a quantity<sup>1</sup> of the material under examination as should contain about 4 grammes of tannin is taken, finely powdered and extracted with successive amounts of hot water at from 45° to 50°. When a material which is difficult to extract, such as wood or bark, is under investigation, the temperature should be from 80° to 90°. Finally the whole is made up to 1 litre in a graduated flask and thoroughly mixed. Of this unfiltered mixture 100 cc. are next taken and treated with 10 grammes of powdered hide which has been slightly chromicized ;<sup>2</sup> for this purpose, 10 grammes of the powder are first soaked in 25 cc. of water for fifteen minutes and the liquid under examination is then added. After thorough stirring the mixture is left for twelve hours, then filtered, and 50 cc. of the filtrate evaporated to dryness in a tared capsule. The residue is then dried to a constant weight at 100°.

The untreated liquid is next filtered (if necessary a little kaolin is first added), care being taken to reject the first 300 cc. of filtrate, since the filter retains a little of the tannin contained in the first portions of the filtrate. A Berkefeld filter connected to a filter-pump is recommended for this filtration.

Fifty cubic centimetres of the clear filtrate are next evaporated to dryness as in the previous case. If the weight of the residue from the extract treated with the powdered hide be  $p$ , and  $p'$  be the weight of this second residue from the untreated extract, then  $p' - (p \times 1.25)$  gives the weight of tannin fixed by the hide, so that the specimen examined contains  $[p' - (p \times 1.25)] \times 20$  grammes of tannin.

<sup>1</sup> For example, 30 to 36 grammes of oak-bark, 15 to 16 grammes of sumac, 32 grammes pine-bark, 6 grammes of oak-gall, etc.

<sup>2</sup> Such powder may be obtained from various tannery schools, *e.g.* at Leeds.

This empirical method is a simplification of that actually employed in the leather industry; it gives very constant results when conducted under rigorously standardized conditions. For these, however, the special technical works must be consulted.

### Estimation of Ethereal Sulphates in the Urine

197. The phenol bodies introduced into the organism and those which are produced there are usually eliminated in the urine as ethereal sulphates ("sulfoconjugués"). The same is true of indole. An idea of the total amount of these bodies may be gathered from an estimation of the sulphuric acid combined with them. To effect this, it is necessary to hydrolyse these compounds by heating with an acid, such as hydrochloric, and to precipitate the liberated sulphuric acid by a salt of barium, the resulting barium sulphate being collected and weighed. The figure so obtained gives the amount of barium sulphate corresponding to the *total* sulphuric acid present in the sample, *i.e.* "free" as well as "ethereal." In a control experiment the free sulphates which occur in the urine are similarly precipitated and weighed, but without any previous hydrolysis. The difference between this figure and the preceding gives the weight of barium sulphate corresponding to the ethereal sulphate present, whence the amount of sulphuric acid can be easily calculated.

198. *Estimation of "Free" and "Ethereal" Sulphuric Acid.*—Into a Bohemian glass beaker of 150 cc. capacity are put 50 cc. of filtered urine, an equal volume of water, and 2 cc. of pure hydrochloric acid (22° B.). The whole is heated on the water-bath for five hours, the beaker being covered with a glass plate to prevent evaporation. Then with constant stirring a 10 per cent solution of barium chloride is added, drop by drop, as long as a precipitate forms (about 5 cc. generally are required), and the whole is allowed to cool. The supernatant liquid is decanted on to a suitable filter. The precipitate remaining in the beaker



is mixed with 100 cc. of water, to which 1 cc. of glacial acetic acid has been added ; the whole is heated a few minutes in the water-bath and then allowed to cool. The liquid is decanted on to the filter as before ; the precipitate is also put on the filter by means of a jet from a wash-bottle. As barium sulphate adheres to the walls of the beaker, the last traces are removed by adding a few cubic centimetres of water and rubbing the sides of the vessel with a glass rod, over the end of which a piece of rubber tubing has been slipped. This operation is repeated two or three times, and the funnel and filter are dried by placing in the oven at 100°. The precipitate is detached from the filter, which is then incinerated and the precipitate again added. The whole is heated to redness, and after cooling the residue is moistened with one or two drops of 10 per cent sulphuric acid, in order to regain as sulphate such sulphides as have been formed by the reducing action of the carbon during heating. Again the whole is evaporated to dryness on the water-bath, and once more it is heated to redness. When cool it is weighed ; hence we obtain the weight of barium sulphate corresponding to the " ethereal sulphates " and " free sulphates " of the urine.

199. *Estimation of " Free " Sulphates in Urine.*—In a complementary experiment the " free " sulphates in the urine are estimated. For this purpose 50 cc. of filtered urine are heated on the water-bath with an equal volume of water and 1 cc. of glacial acetic acid. A 10 per cent solution of barium chloride is added drop by drop until no further precipitate is formed. The whole is then allowed to cool, the supernatant liquid is decanted on to a filter as before, and the precipitate is taken up in 100 cc. of water, to which have been added 2 cc. of pure hydrochloric acid (22° B.). The mixture is heated on the water-bath and constantly stirred, so as to assist the solution of such barium phosphate as may be present ; it is then allowed to cool, and the barium sulphate collected and weighed as before. The weight of barium sulphate formed corresponds to the " free " sulphates of the urine.

By subtracting the figure so obtained from that obtained in the previous section (§ 198), we obtain the figure corresponding to the "ethereal" sulphates. In analytical returns this is expressed in terms of per litre of urine. The weight of barium sulphate multiplied by 0.3432 gives the corresponding weight of  $\text{SO}_3$ .

## CHAPTER IX

### FATS AND GLYCEROL

#### Properties of Fats

200. THE fats may generally be easily recognized by their physical characters. Insoluble in water, soluble with difficulty in strong alcohol (with the exceptions of croton oil and castor oil), they are freely soluble in ether, petrol, benzene, carbon-disulphide, carbon-tetrachloride, chloroform, acetone, and certain other reagents. Those which are liquid (oils), or which melt easily (butter), give persistent translucent marks when applied to paper; this distinguishes them from the so-called "essential oils," which produce marks of similar appearance, but which disappear after a time owing to evaporation.

The naturally occurring fats are always mixtures of different glycerides, some being derived from the unsaturated fatty acids (oleic, linoleic, etc.), while others are derived from saturated acids of the fatty series, principally palmitic and stearic.

201. Two staining reagents are especially used to demonstrate the presence of fats in sections, osmic acid and alkanet. The former is employed in 1 per cent watery solution; it colours the fat droplets an intense black, due to the reduction of the osmic acid by olein, which is generally present in fats. The myelin of nerves is similarly coloured, by reason of the presence in it of oleic residues.

202. To demonstrate fatty materials in vegetable tissues an acetic <sup>1</sup> acid solution of alkanet is used. A section of the

<sup>1</sup> To prepare this solution of alkanet, 10 grammes of powdered alkanet

tissue is covered with the reagent in a watch-glass. The fatty globules, or the droplets of volatile oils, are coloured red. They are easily differentiated by first treating the section with alcohol, which instantaneously dissolves the essential oils, leaving the fats *in situ* (with the exception of the two oils previously mentioned—castor oil and croton oil).

### Formation of Fatty Acids by the Saponification of Suet

203. Ten grammes of suet are melted on the water-bath in a porcelain basin of 250 cc. capacity. When the temperature reaches 90°, add 8 cc. of soda-solution (36° B.) and 5 cc. of 96 per cent alcohol, and stir the mixture for a minute. Next add 200 cc. of boiling water, stir and keep the mixture boiling gently over a flame until complete solution of the soap has occurred; too great frothing should be avoided. Remove about 10 cc. and place 5 cc. in each of two test-tubes. These samples may be used to show (1) that the soap is precipitated by sodium chloride, when not in too dilute solution; and (2) that on the addition of the chlorides of calcium or barium, white insoluble precipitates of calcium or barium soaps are produced. The remainder of the solution is again heated, and ordinary hydrochloric acid is added, drop by drop, until the froth abruptly disappears. The fatty acids separate out in flakes; heat again to boiling for some minutes to collect them in an oily supernatant layer, which on cooling yields a crystalline cake of fatty acids.

### Separation of Unsaturated Fatty Acids

204. The liquid fatty acids, particularly the unsaturated

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root are extracted with alcohol in a glass tube drawn out at its lower extremity, where it is furnished with a cotton-wool plug (not packed too tightly). The alcohol is added in successive small amounts until about 50 cc. of the tincture have been collected. To this is now added 5 cc. of glacial acetic acid and a solution of 32 grammes of chloral hydrate dissolved in an equal weight of water. The mixture is allowed to stand overnight and filtered. (Guignard.)

acids—oleic, linoleic, etc.—give lead salts which are soluble in ether, while those of the solid fatty acids are almost insoluble.<sup>1</sup> A method of separating them has been based upon this property.

Ten grammes of suet are saponified with 10 cc. of soda solution as above (§ 203), but 40 cc. of alcohol are added, to dissolve the soaps. When saponification is complete, a few drops of phenolphthalein are added, and the mixture neutralized by the addition of 10 per cent acetic acid. The liquid is then poured into a solution of neutral acetate of lead (10 grammes in 500 cc. of water), and boiled in a flask of one-litre capacity. A precipitate of lead soaps forms; the flask is now cooled under a stream of water, and the precipitate allowed to settle. The precipitate will be found to adhere to the sides of the flask; the supernatant fluid is decanted as long as it remains clear. The precipitate is washed three times with 100-150 cc. of warm water (50°-60°); it is then drained well, and the small quantity of water remaining is carefully removed with filter-paper. To the soaps are next added 150 cc. of ether, the flask is connected to a reflux condenser, and the contents boiled for fifteen minutes on a water-bath. It is allowed to cool and left overnight. The ethereal solution is next filtered, and dilute hydrochloric acid (1 part strong acid to 4 of water) is added until a precipitate forms; from 50 to 60 cc. of acid are generally required. The ethereal extract, which contains the fluid acids, is decanted, washed once or twice with water, decanted on to a dry filter, and then subjected to distillation on the water-bath. Oleic acid remains behind as a clear yellow oily liquid.

If it is required to avoid all alterations in the unsaturated acids, the solution in ether and the distillation must be conducted in a current of some inert gas, such as carbon-dioxide.

<sup>1</sup> This is not absolutely invariable, since erucic and elaidic acids, which are unsaturated, form lead salts which are only very slightly soluble in cold ether. (Lewkowitsch.)

### Melting-Points of Fatty Acids

205. Although Maquenne's block (§ 96) may be used for the determination of the melting-points of fatty substances, it is better to employ the capillary tube method.

For this purpose a glass tube from 4 to 6 millimetres in diameter is drawn out in the blowpipe, so as to make a thin-walled tube about 1 mm. in diameter and 10 cm. long, and closed at one end. This is cut off a little above the drawn-out portion, and a fragment of the material whose melting-point is to be determined is dropped in; this should pass down as low as the lower third of the tube, which is then fixed to the stem of a thermometer by two rubber rings cut with scissors from a piece of rubber tubing. The thermometer is suspended in a beaker of Bohemian glass of half-a-litre capacity, which is two-thirds filled with distilled water, and containing enough mercury just to cover the bottom of the vessel. The thermometer bulb should be a few centimetres from the bottom of the beaker; the capillary tube is adjusted in such a manner that its upper extremity is out of the water, while the portion containing the fragment of material under observation is situated midway in the length of the bulb. The beaker is of course supported so that it can be heated from below. To avoid over-heating of its walls it is advisable to stand it upon a sheet of wire gauze, with a diameter about 4 cm. greater than that of the beaker. A good plan also is to raise the water in the vessel to boiling before commencing the experiment, and then to allow it to cool; the formation of bubbles upon the walls of the beaker and the thermometer bulb is thereby avoided.

When the apparatus is finally adjusted, it is gently heated, so that the thermometer rises by about  $1^{\circ}$  per minute when it is in the neighbourhood of the required melting-point. When this is reached the material in the capillary tube becomes transparent, and the temperature is noted.

The figure obtained may be corrected for the part of the

stem which is not immersed; this is, however, unimportant for temperatures below  $60^{\circ}$ - $70^{\circ}$ .

If the acids or fats are of a pasty consistency, it is impossible to drop a particle into the capillary tube. In such cases a small quantity may be melted in a capsule and the end of the capillary tube (of course in this case left open) plunged into it. The melted substance ascends in the tube by capillarity. As small a quantity as possible is taken up in this way, the end of the tube is closed, the substance is allowed to solidify completely (this may take several hours), and the experiment conducted as before.

### Reactions of Glycerol

206. *Formation of Acrolein*.—Put a pinch of powdered potassium bisulphate in a test-tube, allow a drop of glycerol to fall upon it, and warm until white fumes are evolved. The irritating odour of acrolein will then be noticed (the tube should be put away in a fume chamber).

The vapour evolved reduces an ammoniacal solution of silver nitrate.<sup>1</sup> Moisten the end of a glass rod and introduce it into the lumen of the tube, when a black colour will show the presence of metallic silver. Since this reduction is favoured by heat, the reagent should be heated to boiling before the rod is dipped in it.

207. *Reaction of Glycerol with Borax*.—A solution of borax which is faintly alkaline to litmus becomes definitely acid upon the addition of glycerol,<sup>2</sup> owing to the formation of a glyceroborate, a compound which also has the power of imparting a green tint to the colourless Bunsen flame.

To produce this latter reaction, a platinum loop (or a loop of fine iron wire) is plunged into glycerol, a fragment

<sup>1</sup> Ammoniacal silver nitrate is prepared by mixing equal volumes of 1 or 2 per cent silver nitrate solution, strong ammonia and caustic soda solution.

<sup>2</sup> Reducing sugars and mannitols give the same reaction. To establish definitely the presence of glycerol, advantage is taken of the fact that it is volatile in a current of steam. The test can then be applied to the distillate so obtained.

of powdered borax is next taken up upon it, and the whole brought *close to* the base of the flame.

208. *Colour Reactions of Glycerol*.—Glycerol when oxidized by bromine water gives a mixture of reducing substances which have characteristic colour reactions (Denigès).

To obtain these, 1 cc. of a 5 per cent watery solution of glycerol and 10 cc. of bromine water (3 cc. bromine to 1 litre water) are put in a test-tube and boiled on the water-bath for twenty minutes. Excess of bromine is then completely removed by boiling and the mixture allowed to cool.

To 5 cc. of this cooled liquid in a test-tube are added 2-4 drops of a 5 per cent alcoholic solution of codeine and 2 cc. of pure sulphuric acid. The mixture is heated on the boiling water-bath for two or three minutes, when a beautiful greenish-blue colour is obtained.

By proceeding in the same way, only substituting a 5 per cent alcoholic solution of resorcinol for the codeine, a fine wine-red colour is obtained without heating.

209. *Detection of Glycerol in Wine*.—These reactions may be applied to the detection of glycerol in wine. Ten cubic centimetres of wine are decolorized by the addition of 1 cc. of lead subacetate solution. The mixture is filtered and to the filtrate is added a little powdered sodium sulphate to precipitate excess of lead. It is again filtered and 1 cc. of the filtrate is heated with 10 cc. of bromine water as before (§ 208), and the tests with codeine or resorcinol applied.

Wine which has not been decolorized may also be used. In this case, 1 cc. is evaporated to a syrup on the water-bath, which is then heated with 5 cc. of *saturated* bromine water. The subsequent procedure is as before; more bromine is necessary in this case, as a portion is used up in precipitating the tannin-like substances present in the wine.

### Determination of the Index of Saponification

210. The index of saponification (or Köttstorfer's index) of a fat is expressed by the number of milligrammes of KOH



necessary to saturate the fatty acids contained in one gramme of the material.

To determine this index a conical flask of 150 cc. capacity is accurately weighed, and about 5 cc. of the oil (castor oil, olive oil, etc.) or of melted fat (butter, suet, etc.) are introduced. The flask and contents are accurately re-weighed, to get the weight  $p$  of the added fat. Next 25 cc. of a "normal" solution of alcoholic potash<sup>1</sup> are added, the mouth of the flask is covered with a watch-glass and the whole kept at 90° on a boiling water-bath for twenty minutes, with frequent shaking. The entire disappearance of all unchanged fat is assured by the addition of a little water, which should produce no turbidity. It is then allowed to cool, a drop of phenolphthalein added, and titrated with normal sulphuric acid until the red colour just disappears.

A control experiment is done by titrating 25 cc. of the potash solution with the normal acid; by difference we find the number of cubic centimetres of potash solution corresponding to the fatty acids ( $n$ ); the required index is then given by the formula:

$$\frac{n \times 56}{p}.$$

### Determination of the Iodine Index

211. The unsaturated fatty acids (*e.g.* oleic acid) and their corresponding glycerides can fix as many iodine molecules as they possess double linkages. The iodine index (or Hübl's index) is expressed by the number of grammes of iodine absorbed by 100 grammes of the fatty material.

For this purpose about .3 gramme (if a drying oil) or .5 gramme (if a non-drying oil) is weighed out correctly to a milligramme, in a watch-glass. This is dissolved in 15-20 cc. of chloroform and introduced into a half-litre flask provided with a ground stopper. An equal volume of chloroform is

<sup>1</sup> To prepare normal alcoholic potash 70 grammes of potassium hydrate are dissolved in 25-30 cc. of warm water. The mixture is made up to 1 litre with alcohol. When the liquid has settled, the clear portion is decanted off.

put into a similar control flask. To each flask are added exactly 20 cc. of a 5 per cent solution of iodine<sup>1</sup> in 95 per cent alcohol and 20 cc. of a 6 per cent solution of mercuric chloride, also in alcohol (95 per cent).

The flasks are stoppered and shaken, then left to stand for two hours. At the end of this time 25 cc. of a 20 per cent solution of potassium iodide are added to each flask; they are shaken for a minute or two, and the contents then titrated with N/5 sodium thiosulphate solution.<sup>2</sup> This is run in until the yellow colour has almost disappeared and then added, drop by drop, until it has completely vanished. The difference  $v - v'$  between the volumes of thiosulphate run into the control and experimental flasks, expressed in cubic centimetres and multiplied by  $\cdot 0127 \times 2$ , represents the amount of iodine absorbed. If  $p$  is the number of grammes of fatty material taken, then the iodine index is given by the formula:

$$\frac{v - v'}{p} \times \cdot 0254 \times 100.$$

### Estimation of Fats in Seeds

212. The procedure is the same whether the seed contains a large amount of oil (as in colza, poppy-seed, earth-nut, castor-oil bean, almond) or fat (as cocoa-nibs, laurel, nutmeg), or whether it contains a small quantity only (coffee, maize).

The seed is ground up in a mill, and a specimen weighing from 1-10 grammes is dried in the hot-air oven in a small capsule. After an hour or two at  $105^{\circ}$ - $110^{\circ}$ , the specimen is transferred to a little glass funnel furnished with a plug of absorbent cotton-wool. The capsule is washed out two or three times with ether, carbon-disulphide, petrol or

<sup>1</sup> The iodine solution must be prepared in the cold. To obtain rapid solution of the iodine, it is put in a little gauze sachet which is suspended by a thread in the upper part of the flask.

<sup>2</sup> To prepare the standard thiosulphate solution, exactly 496 grammes of the dry crystallized salt are dissolved in a little cold water. The volume is made up to 1 litre in a graduated flask. The formula of the crystallized salt is  $\text{Na}_2\text{S}_2\text{O}_3 + 5\text{H}_2\text{O}$  and its molecular weight 248. The above-named quantity then gives a N/5 solution. Photographic "hypo" crystallizing in little prisms like crystalline sugar answers perfectly well.

chloroform, the washings being poured into the funnel to exhaust the ground-up seeds. This washing is continued until a small quantity of the extract (say 1 cc.) gives no residue when evaporated in a watch-glass. The filtrate is then evaporated on a water-bath<sup>1</sup> in a weighed glass beaker with vertical sides. When a constant weight has been obtained, the weight of fatty matter is noted and reported as so much per 100 grammes of seed.

To obviate loss of material through the liquid creeping up the sides of the vessel during evaporation on the water-bath, the latter must have vertical sides which are sufficiently high (2-3 centimetres). The evaporation may be accelerated by allowing a current of air to play on the surface of the liquid; this may be effected by placing the mouth of a small funnel just above it and connecting up with a filter-pump.

### Estimation of Butter Fat in Milk

213. The following method is applicable both to fresh milk and to milk which has undergone spontaneous coagulation.

Ten grammes of milk<sup>2</sup> are weighed out in a thin glass beaker, and sufficient 1.5 per cent solution of soda added to dissolve the casein; the quantity necessary manifestly varies with the acidity of the milk. The mixture is transferred to a separating funnel, and the beaker washed out with 5-10 cc. of alcohol and then once or twice more with the same volume of ether. The washings are to be carefully transferred to the separating funnel, which is now stoppered and shaken gently, so as to avoid the formation of an emulsion. Fatty matters are taken up by the ether and when separation is complete the lower layer, containing the casein, is allowed to run out

<sup>1</sup> Note caution, p. xxx.

<sup>2</sup> Milk which has been kept for some days or which has partially clotted is no longer homogeneous, the fatty matter having almost entirely separated. Simple shaking will not suffice to renew the uniform suspension of fat. To render it homogeneous again, after having dissolved the casein in 1.5 per cent soda solution, a little saponin (0.04-0.05 gm. per litre) is added. The mixture is warmed to 40° for a few minutes and then shaken. (A. Frouim.)

and is collected in a beaker. The ethereal extract is washed with a few cubic centimetres of water; this washing is added to the casein solution, while the ether-extract is decanted (from the upper end of the separating funnel) into a tared glass beaker.

The alkaline solution of casein is again extracted with 15-20 cc. of ether as before; the ethereal extract is mixed with that previously obtained and the whole evaporated to a constant weight as described in § 212.

The solution of casein may be used for the estimation of the amount of casein in milk (see § 357).

### Estimation of Fats in Animal Tissues

214. The fats of animal tissues cannot be extracted completely by simple exhaustion with fat solvents; a preliminary decomposition of the material by alkalis is necessary (Kumagawa and Suto).

By this procedure the fats and the esters of cholesterol are saponified, and a mixture of alkali salts of fatty acids and free cholesterol is obtained.

215. *Solution of the Material and Extraction.*—The organ examined (liver, kidney, muscle, etc.) is very finely minced, and from 5 to 10 grammes of the pulp are accurately weighed out. This is put in a Bohemian glass beaker with 5 cc. of water and 15 cc. of soda (36° B.). The whole is now heated on the water-bath, care being taken to see that the beaker is placed well down in the boiling water and that it is covered with a watch-glass to prevent undue evaporation. At the end of two hours, during which the mixture has been stirred occasionally, solution will be found to be complete.

It is allowed to cool a little, and then turned into a separator of about 250 cc. capacity, the beaker being washed out three times with a little warm water. The washings are, of course, added to the contents of the separator. Then, drop by drop, diluted hydrochloric acid (1 of acid to 3 of water) is run in until the reaction is slightly acid. Generally about 50 cc. are required, and the apparatus is cooled during

the operation if necessary. After complete cooling, an equal volume of ether is added to the mixture, and the whole thoroughly mixed. When the two layers have separated, the lower is drawn off with the exception of the somewhat turbid last portions. These are allowed to remain in the separator.

The ethereal layer, slightly brownish in colour, is decanted as completely as possible into a flask. The small quantity of turbid fluid remaining contains fatty matters. It is treated with 5 cc. of normal soda to redissolve the precipitate, and shaken with 25-30 cc. of ether. The acid liquid separated in the first extraction is added and the whole again shaken. The reaction is now acid and the free fatty acids are taken up by the ether. The ethereal layer is carefully separated and added to the first ethereal extract.

The ether is now evaporated off on the water-bath, and the residue, dissolved in a *little* dry ether, is filtered in a funnel furnished with a tampon of absorbent cotton-wool. The funnel and wool are washed with a few drops of ether.

The ether extract is dried for a few hours at 40-50°, and then treated with 20 cc. of petroleum-ether. The petroleum-ether extract is filtered as above and the filtrate collected in a glass capsule with vertical sides. It is evaporated to dryness and then dried to a constant weight at 50°<sup>1</sup> for a few hours. Thus we have the combined weights of fatty acids and of non-saponifiable substances, the latter comprising especially cholesterol.

216. *Separation of Non-Saponifiable Substances.*—After obtaining the weight of the dry petroleum-ether extract, it is dissolved again in 30-50 cc. of petroleum-ether, the solution placed in a separating funnel, and 150-200 cc. of N/5 solution of potash<sup>2</sup> in absolute alcohol added. The whole is gently shaken and a volume of water added which is equal to the amount of alcoholic potash solution used. The liquids are mixed and then allowed to stand, when two

<sup>1</sup> By raising the temperature above this there would be a danger of oxidation.

<sup>2</sup> Twelve grammes of caustic potash to the litre of alcohol.

layers are formed. The upper layer contains non-saponifiable substances dissolved in petroleum-ether; the lower, a solution of soaps of fatty acids in 50 per cent alcohol. This lower layer is drawn off and again extracted with 20-30 cc. of petroleum-ether. The two petroleum-ether extracts are mixed, and the liquid distilled off. The residue is treated with 5 cc. of warm alcohol, and 5-10 drops of the  $N/5$  solution of alcoholic potash added; it is then evaporated on the water-bath and dried in the hot-air oven at  $100^{\circ}$  for half an hour. The residue is again extracted with 10 cc. of petroleum-ether, and filtered as before. The funnel and plug are washed twice with 5 cc. of petroleum-ether, and the filtrate evaporated to dryness in a weighed glass beaker. The residue is dried at  $100^{\circ}$  to a constant weight.

Thus the weight of cholesterol is obtained; by deducting this from the total weight previously found (§ 215), we have the weight of fatty acids contained in the fats of the original tissue.

This method is not applicable directly to blood or serum. In these cases, 10-20 grammes of the material are coagulated by alcohol, and extracted with boiling alcohol; the alcoholic extract is then treated with soda, etc., according to the directions in §§ 215-216.

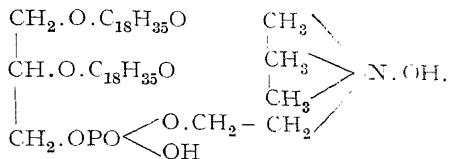
## APPENDIX

### Lecithins

217. Fats are often accompanied by a series of bodies which possess much the same general properties, but are characterized by their richness in phosphorus and nitrogen. To such bodies the name of phospholipines has been given, and they are classified according to the number of atoms of phosphorus and nitrogen which they contain.

Among the most widely distributed are the lecithins, which are compounds of glycerophosphoric acid and choline, in which

the two free hydroxyl groups of the glycerol are combined with two molecules of fatty acid—stearic, palmitic or oleic. The stearic acid compound is thus :



Ordinary lecithin is found in abundance in the yolk of egg (10-11 per cent), and in the brain and spinal cord (10-17 per cent).

### Preparation of Lecithin from Egg-Yolk

218. To extract lecithin from egg-yolk, the yolk is thoroughly freed from the "white," beaten up with 20 cc. of acetone, and decanted on to a filter; this process is repeated until a colourless acetone-extract is obtained. The residual white powder is pressed between filter-paper and then ground up with 20 cc. of chloroform. The acetone dehydrates the yolk, dissolves colouring matter and fats, but does not dissolve lecithin, which is, however, dissolved by chloroform. The chloroform-extract is filtered off and the residue again extracted with 10 cc. of chloroform. The combined chloroform-extracts are introduced into a flask of about 100 cc. capacity, and the chloroform distilled off on the water-bath. The residue, which is syrupy and nearly colourless, consists of crude lecithin. The remaining chloroform is removed by drawing a current of air through the flask.

Lecithin can be identified by recognizing the products of its hydrolysis—phosphoric acid and choline. The residue from the chloroform extractions consists of ovovitellin (see § 340).

### Hydrolysis of Lecithin and Identification of Choline

219. The lecithin obtained as above is placed in an evaporating basin of 100-150 cc. capacity and to it are added 5 cc. of soda-solution (36° B.) and 20 cc. of water. It is next boiled for five minutes and 4 cc. of glacial acetic acid are added. The mixture is well stirred (making sure that it is acid) and boiled for a minute. It is allowed to cool and filtered on a moistened filter. A drop of the filtrate is placed on a glass slide, and two drops of a solution of iodine (iodine 5 grammes, potassium iodide 10

grammes, water 100 cc.) are added. The preparation is examined under a low power of the microscope. An ochre-coloured precipitate is seen, which soon changes to brown opaque crystals ("crystals of Florence," fig. 25).

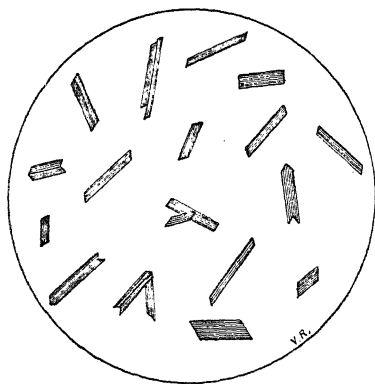


FIG. 25.—Crystals of Florence.

These crystals, consisting of iodhydrate of choline iodide, disappear in a few moments. Their identification, however, indicates the presence of choline.

In the remaining filtrate, phosphoric acid may be identified, either as ammonio-magnesium phosphate or as ammonium phosphomolybdate (§§ 13-14).



## CHAPTER X

### ESSENTIAL OILS AND TERPENES

220. THE so-called essential oils are mixtures of several volatile bodies, and consequently can be distilled over in a current of steam; they usually contain a terpene among their chief constituents. According to the essential oil examined, very varied bodies are met with, such as alcohols, aldehydes, phenols, etc. Essential oils are generally contained in glands of relatively large size; a good example can be seen on examining a thin slice of lemon or orange peel. On paper they leave a mark like a spot of grease, but this disappears on evaporation.

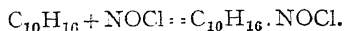
The principal terpenes of the formula  $C_{10}H_{16}$  are pinene (from oil of turpentine) and limonene or citrene (from oil of lemon, bergamot, etc.). Similarly may be mentioned phellandrene (oil of fennel), and among the sesquiterpenes,  $C_{15}H_{24}$ , caryophyllene (oil of clove).

#### Extraction of an Essential Oil by Steam Distillation

221. Into a large flask (2-3 litres capacity) put the vegetable matter (lemon-peel, anise seed, cinnamon, eucalyptus or lavender leaves, turpentine, etc.) to be extracted. This should be broken up as finely as possible and a sufficient quantity of water added to at least half-fill the flask, which is then connected to the reflux condenser of a Schloesing-Aubin apparatus and distilled. The first portions of the condensed liquid consist of water containing droplets

of essential oil; continue the distillation until the droplets disappear. Leave the distillate in a stoppered bottle for a day or two and then decant the separated essential oil. By redistilling the watery residue, a further supply of the volatile extract can be obtained and separated as before.

*222. Reactions of Pinene.*—The majority of terpenes, which are unsaturated bodies, give with nitrosyl chloride a characteristic addition product; thus with pinene (from oil of turpentine) we have,



Mix in a test-tube 3 cc. of oil of turpentine, 4 cc. of amyl nitrite, and 7 cc. of glacial acetic acid; put this in a freezing mixture of ice and salt. When the mixture is thoroughly cold, add drop by drop (stirring continuously meanwhile) 7 cc. of a mixture of equal volumes of glacial acetic acid and strong hydrochloric acid. From the greenish-blue liquid there gradually separates a white crystalline powder of the nitrosochloride, which can be collected on a filter and washed with a little alcohol. This substance, dissolved in chloroform and reprecipitated by methyl alcohol, melts at  $103^{\circ}$ .

### Estimation of Esters in Oil of Lavender

*223.* A certain number of essential oils (lavender, bergamot, etc.) contain esters which can be estimated on saponification by a method analogous to that used for fatty bodies (see § 172). On account of the volatile character of the oil, the treatment with potash is carried out in a flask furnished with a reflux condenser.

*224.* Introduce into a flask of 100 cc. capacity 4 cc. of oil of lavender and determine its weight accurately. Add 10 cc. of alcoholic potash solution of approximately "normal" strength. Fit the mouth of the flask with a cork through which passes a long straight tube to act as condenser, and heat on the water-bath. After half an hour, allow it to cool, add 50 cc. of alcohol and a few drops of

phenolphthalein, and then run in "normal" sulphuric acid till the red tint disappears. Then titrate with the "normal" acid 10 cc. of the original potash solution used. The difference between the two readings gives the quantity of potash which has been used up in neutralizing the acids combined in the esters of the oil of lavender. Hence the amount of ester (in this case linalyl acetate) contained in the volatile oil can be calculated.

Each cubic centimetre of N/1 sulphuric acid corresponds to .196 gramme of linalyl acetate: the findings are expressed with reference to 100 grammes of the volatile oil.

It is evident that if the volatile oil analysed is acid to start with, it is necessary to determine the amount of potash necessary to neutralize it, and to make the required allowance in the calculation.

#### PRINCIPAL ESTERS CONTAINED IN VOLATILE OILS

Esters.	Oil of
Methyl anthranilate $C_6H_4.NH_2.COOCH_3$	Jasmine, orange, tuberose.
Methyl salicylate $C_6H_4.OH.COOCH_3$	Gaultheria, meadow-sweet.
Geranyl acetate $CH_3.COO.C_{10}H_{17}$	Palma-rosa.
Linalyl acetate $CH_3.COO.C_{10}H_{17}$	Lavender, bergamot, neroli.
Bornyl acetate $CH_3.COO.C_{10}H_{17}$	Leaves of conifers, thyme.
Terpinyl acetate $CH_3COOC_{10}H_{17}$	Eucalyptus, niaouli.
Menthyl acetate $CH_3.COOC_{10}H_{19}$	Peppermint.
Santalyl acetate $CH_3.COO.C_{15}H_{25}$	Sandalwood.
Estragol $C_6H_4.C_3H_5OCH_3$	Tarragon.
Anisol $C_6H_4.C_3H_5OCH_3$	Anise, fennel, star-anise.

#### Estimation of Alcohols in Oil of Peppermint

225. To estimate the alcohols present in an essential oil, it is first treated with acetic anhydride, which transforms the alcohols into their corresponding acetic esters.

226. Heat in a 100 cc. flask fitted with a reflux condenser, 10 cc. of oil of peppermint with 10 cc. of acetic anhydride and 2 grammes of anhydrous fused sodium acetate. After boiling from an hour to an hour and a half, the menthol is converted into its acetic ester. Allow the mixture to cool, and treat with water to remove excess of the

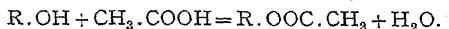
reagent. For this purpose, add about 50 cc. of water and a few drops of litmus; stir, and while stirring run in, little by little, a solution of sodium carbonate until the liquid is just permanently alkaline. Decant the contents of the flask into a separating funnel, draw off the watery liquid and collect the acetylated extract in a little bottle containing a few grammes of anhydrous sodium sulphate. (Anhydrous sodium sulphate is prepared by desiccating a few grammes of the crystals in a porcelain capsule over a naked flame, until they are all converted into a white opaque powder.)

Shake vigorously to ensure desiccation, and filter upon a *dry* filter. Remove the required weight of the filtered acetylated compound upon which the determination is to be made (say 3-4 grammes) and proceed to the titration of the esters as shown in § 224, using in this case, however, 20 cc. of alcoholic potash instead of 10.

The amount of "normal" sulphuric acid needed in the subsequent titration corresponds not only to the potash used to saponify the acetic ester, but also to that which has been used in saponifying esters normally present. This last figure must be obtained by a preliminary experiment, and deducted from the total figure. But this is not all. The weight of the acetylated product must be diminished by the weight of the acetyl radicle which it contains; it may be noted that each cubic centimetre of "normal" sulphuric acid corresponds to .156 gramme of menthol as well as to .043 gramme of acetyl.

If  $p$  = the weight of the acetylated compound and  $n$  the number of cc. (corrected) of sulphuric acid used, then the real weight of the essential oil is given by  $p - (n \times .042)$ ,<sup>1</sup> and the percentage of alcohol by  $\frac{n \times .156 \times 100}{p - (n \times .042)}$ .

<sup>1</sup> The reaction takes place according to the equation



The acetyl group replaces the H of the hydroxyl. From the weight obtained as above ( $p$ ) must accordingly be subtracted

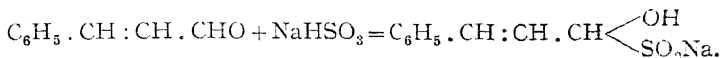
$$\frac{n \times (43 - 1)}{1000} \text{ or } n \times .042.$$

## PRINCIPAL ALCOHOLS CONTAINED IN ESSENTIAL OILS

Alcohols.	Essential Oil of
Geraniol $C_{10}H_{18}O$	Geranium, rose, citronella, palma-rosa.
Linalol „	Lavender, neroli, bergamot.
Borneol „	Borneo - camphor, citronella, rose-mary.
Terpeneol „	Niaouli, eucalyptus, valerian.
Rhodinol $C_{10}H_{20}O$	Rose, geranium.
Citronellol „	Citronella.
Menthol „	Peppermint, pennyroyal.
Santalol $C_{15}H_{26}O$	Sandalwood.

## Estimation of Cinnamic Aldehyde in Oil of Cinnamon

227. This estimation is based upon the fact that cinnamic aldehyde forms a compound with sodium bisulphite which is soluble in water ; this is not the case with the other constituents of the volatile oil.



By measuring the quantity of essential oil undissolved after treatment, the amount of aldehyde can be estimated.

228. Take a flask of 150 cc. capacity, with a long narrow neck graduated in tenths of a cubic centimetre, and run in with a pipette 10 cc. of the essential oil. Add an excess of sodium bisulphite in concentrated solution (say 50 cc. of the commercial solution at 36° B.). Shake, to facilitate the formation of the bisulphite-aldehyde compound which separates in solid form ; add 50 cc. of water and heat on the water-bath, with frequent shaking to ensure solution and the setting free of the terpene residue. Add water so as to bring this residue into the graduated part of the neck of the flask, cool and read off the volume  $n$  of the supernatant residue. The percentage of the aldehyde is given by  $(10 - n) \times 10$ . A similar method may be adopted for the estimation of benzaldehyde in oil of bitter almonds.

## PRINCIPAL ALDEHYDES CONTAINED IN ESSENTIAL OILS

Aldehydes.	Essential Oil of
Citral $C_{10}H_{16}O$ . . . . .	Citron, lemon, balm.
Citronellal $C_{10}H_{18}O$ . . . . .	Citronella, lemon, lemon-grass.
Benzoic Aldehyde $C_6H_5.CHO$ . . . . .	Bitter almonds.
Cinnamic Aldehyde $C_6H_5.CH:CH.CHO$ . . . . .	Cinnamon.
Cumic Aldehyde $C_6H_4.C_3H_7.CHO$ . . . . .	Cummin.
Salicylic Aldehyde $C_6H_4.OH.CHO$ . . . . .	Meadowsweet.
Anisic Aldehyde $C_6H_4.OCH_3.CHO$ . . . . .	Star-anise, fennel.

## Estimation of Phenols in an Essential Oil

229. This is based upon the solubility of phenols (thymol, eugenol, etc.) in watery solutions of potash or soda, which do not dissolve the terpene compounds. It must, however, be borne in mind that alkaline solutions, if too concentrated, may dissolve other constituents than the phenols; hence a solution of soda stronger than 5 per cent may not be used for the estimation of phenols in this connection (*e.g.* for oil of thyme). For oils containing eugenol a strength of 3 per cent should not be exceeded.

230. *Estimation of Thymol in Oil of Thyme.*—Introduce into a flask of 150 cc. capacity with a graduated neck, as described above (§ 228), 10 cc. of soapmaker's lye, 80 cc. of water and 50 cc. of the essential oil. Shake, and allow to stand. When the undissolved oil has completely separated, add enough 5 per cent soda-solution to bring the line of demarcation between the two layers of liquid into the graduated part of the neck. The diminution in volume represents the amount of thymol originally present; the thymol can be liberated from its alkaline solution by the addition of sulphuric acid in slight excess and allowing it to stand overnight, when it will have risen to the surface of the liquid.

231. *Estimation of Eugenol in Oil of Clove.*—Proceed exactly as in the case of oil of thyme, using 6 cc. of soap-

maker's lye, 80 cc. of water, and 5 cc. of the essential oil; the subsequent addition of alkali is made with 3 per cent soda-solution.

#### PRINCIPAL PHENOLS CONTAINED IN ESSENTIAL OILS

Phenol.	Oil of
Thymol $C_6H_3 \cdot CH_3 \cdot C_3H_7 \cdot OH$ . . .	Thyme, wild thyme, ajowan.
Carvacrol " " . . .	Marjoram, savory, thyme.
Eugenol $C_6H_3 \cdot C_3H_5 \cdot OCH_3 \cdot OH$ . . .	Clove, cinnamon.

## APPENDIX

### CHOLESTEROL AND BILE ACIDS

#### Reactions of Cholesterol

232. Cholesterol,  $C_{27}H_{46}O$ , is a terpene derivative having the properties of a tertiary alcohol, and generally accompanies fatty materials in the living organism. It is present in the bile and is a frequent constituent of gallstones; it is soluble in the ordinary solvents for fats and can be separated from them by the action of alkalies, which convert the fats into soaps which are soluble in water, while the cholesterol is unattacked and remains insoluble. The following are the main characteristic reactions of cholesterol.

233. Dissolve a fragment of the substance (*e.g.* of gallstone), about twice the size of a pin's head, in 2-3 cc. of chloroform, with slight heating if necessary; then mix with an equal volume of pure strong sulphuric acid. The chloroform assumes a blood-red colour and the acid has a greenish fluorescence. On adding 5-6 cc. of glacial acetic acid, this latter acquires a rose colour with a very marked green fluorescence (Salkowski).

234. On dissolving a few crystals of cholesterol in 1 cc. of chloroform and adding 1 cc. of acetic anhydride, then adding concentrated sulphuric acid, drop by drop, a play of colours is obtained—rose, violet, blue, and green. The first tint (rose) is produced by a single drop of the acid and rapidly passes into violet. The blue appears after a short interval, while the production of the green tint requires a further addition of acid.

235. Dissolve a fragment of the substance supposed to contain cholesterol in 1-2 cc. of alcohol, warming carefully meanwhile. Put two or three drops of the solution upon a glass slide and

allow them to evaporate. Upon microscopic examination cholesterol is seen to form rhombic plates, of which one angle is often deficient. On the addition of a drop of sulphuric acid (1 part water to 5 parts acid) and gentle heating, the edges of the plates are coloured first red and then violet.

236. Evaporate 2 cc. of a chloroform solution of cholesterol in a small capsule, moisten the residue with hydrochloric acid containing a trace of ferric chloride (yellow commercial hydrochloric acid answers the purpose), and evaporate again to dryness. Cholesterol produces a violet colour.

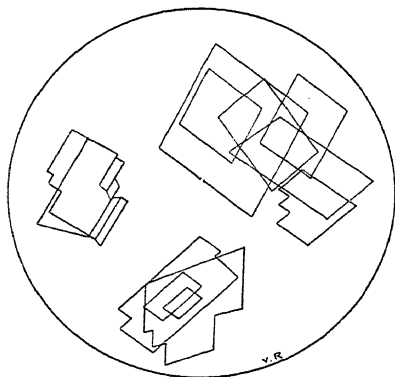


FIG. 26.—Cholesterol.

### Reactions of Bile Acids

237. The bile acids—glycocholic and taurocholic acids—are compounds of cholalic acid, which is a complex terpene derivative, with glycoll and taurine. They exist in the bile as alkali salts, which have an extremely bitter taste. To recognize them, Pettenkofer's reaction may be used; this consists in the production of a rose-purple colour with cane sugar and sulphuric acid. Since this colour may be given by substances other than cholalic acid, it is advisable to examine the spectrum.

238. Put into a test-tube 3 cc. of water and carefully run in 6 cc. of strong sulphuric acid, shake gently, and cool under the tap. Then add 6 drops of a 10 per cent solution of lævulose and a drop of fresh bile; shake again. If the temperature is about 25-30°, after about five minutes a pale rose colour appears which gradually deepens; after ten minutes it has become violet and looks like a dilute solution of permanganate. Spectroscopically,



a deep broad band is seen in the green (between w.l. 500 and 530) and a second narrow band in the yellow (about w.l. 575). These bands gradually enlarge and the whole spectrum becomes invaded except the red, according to the depth of colour attained.

239. To see these two bands conveniently, note that the slit of the spectroscope is not too wide, but get at the same time a bright distinct spectrum; adjust the instrument as for observing the black lines of the solar spectrum, so that the D line corresponds with w.l. 590 on the scale; the line E is then about w.l. 530.

In the above, saccharose can be used instead of lævulose, but then the development of colour is slower. According to Ville

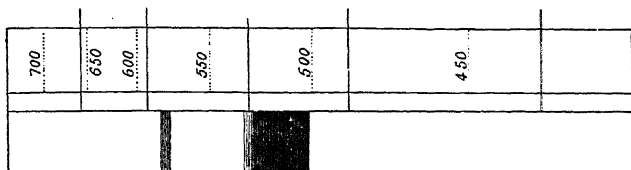


FIG. 27

and Derrien the reaction is due to the presence of methyloxy-furfurol, produced by the action of the acid on the lævulose.

240. The bile acids and their salts markedly diminish surface tension. This may be demonstrated by half-filling a test-glass with water, adding a little bile, and mixing. If a little finely powdered charcoal or flowers of sulphur is lightly thrown on to the surface, it almost immediately sinks. If thrown on to pure water, on the other hand, both will remain a long time on the surface without becoming moistened. By this means 1 part in 10,000 of bile acids can be demonstrated.

### Estimation of Cholesterol in the Tissues

241. The cholesterol existing in tissues, mainly in the condition of various ethers, may be liberated by soda and extracted with a suitable solvent. It can be estimated along with the fatty acids, as described in § 215. If it is desired only to estimate the cholesterol, the procedure may be simplified as follows.

242. Heat 5-10 grammes (finely divided) of the organ to be studied with 15 cc. of water and 15 cc. soda-solution (36° B) on the water-bath. The vessel must be well plunged into the boiling

water and covered to prevent evaporation. After two hours add 20 cc. of water, mix, and allow to cool. Extract with 60 cc. of ether in a separating funnel. Collect the ether in a flask; again extract with 30-40 cc. of ether, and mix the extracts. The ether is distilled off on the water-bath and the residue, completely dried, is taken up with 10 cc. of alcohol, to which a single drop of soda-solution has been added. Evaporate on the water-bath and dry in the oven at 100° for half an hour. Extract the residue with 10-15 cc. of petroleum-ether, filter, and collect the filtrate in a tared dish with vertical sides; wash the filter two or three times with petroleum-ether and evaporate at 100° to constant weight; the crystalline residue consists of cholesterol.

## CHAPTER XI

### ALKALOIDS

#### Precipitation Reactions of the Alkaloids

243. ALMOST all alkaloids of vegetable origin when in watery solution give precipitates which are only very slightly soluble with the following reagents :

Solution of iodine in potassium iodide	Brown ppt.
Potassio-mercuric iodide . . . . .	White ppt.
Silicotungstic acid . . . . .	White or pale ppt.

The medium must, however, always be slightly acid (one or two parts of sulphuric or hydrochloric acids per 1000). Most alkaloids are also precipitated by the following, but their solutions must be rather more concentrated than in the first instance.

Picric acid . . . . .	Yellow ppt. often crystalline.
Mercuric chloride . . . . .	White ppt.
Platinic chloride . . . . .	Yellow ppt.
Tannic acid, potassium ferrocyanide (in presence of acetic acid), etc.	

In addition, those alkaloids which are insoluble in water are precipitated from solutions of their salts by ammonia. The precipitate is usually white and amorphous, and insoluble in excess of the reagent.

Morphine, however, gives a crystalline precipitate easily soluble in excess of ammonia.

The precipitation reactions of the alkaloids may be

studied with 1-2 per cent solutions of the sulphates of quinine and strychnine, or morphine hydrochloride.

### Colour Reactions of the Alkaloids

244. Certain alkaloids give characteristic colour reactions. Examples are quinine, cinchonine, strychnine, brucine, morphine, codeine, atropine, and cocaine.

*Quinine*.—Note the blue fluorescence characteristic of quinine salts in solution with sulphuric acid, which in itself is an important test. Quinine salts give a very sensitive colour reaction known as the thallioquinine reaction. In order to obtain it proceed as follows. To 5 cc. of a cold saturated solution of quinine sulphate, in a test-tube, add 6 drops of saturated bromine water ; this quantity is enough to give the mixture a light yellow colour, but without producing a permanent precipitate. On then adding ammonia (diluted with two or three times its volume of water) and shaking, a fine red colour is produced which is evanescent. On adding more ammonia the red passes immediately to an emerald green. This last colour is stable. Deficiency or excess of bromine water tends to inhibit the reaction. Either the red or green solution when shaken up with amyl alcohol gives up its colour to the alcohol.

245. *Cinchonine*.—Watery solutions of cinchonine or its salts, *slightly* acidulated with 10 per cent sulphuric acid, give on addition of a few drops of a 10 per cent solution of potassium ferrocyanide a yellow precipitate. On warming to 50° or 60° the precipitate dissolves, and on cooling deposits beautiful golden yellow needle-like crystals.

246. *Strychnine*.—If a trace of strychnine is dissolved in a fair-sized drop of strong sulphuric acid, placed in the middle of a white porcelain capsule, and a minute amount of powdered potassium bichromate is added, each fragment of the salt dissolves, giving a violet colour ; on stirring, the whole acquires a fine purple colour, which changes to a brownish-red.

247. *Brucine*.—Dissolve a particle of brucine placed in

a watch-glass in a drop of strong nitric acid ; a blood-red colour is produced, which on addition of a solution of stannous chloride changes to violet. Morphine and codeine also give a red colour with nitric acid, but the colour does not change upon addition of stannous chloride.

248. *Morphine*.—As morphine contains a phenol group, it gives a blue colour with ferric chloride if the solution is not too dilute.

249. It has a marked reducing action. If a few drops of a solution of a salt of morphine are added to 2 cc. of a 10 per cent watery solution of iodic acid, the liquid becomes yellow. Upon shaking with 1 cc. of chloroform, this becomes violet, due to the solution of the liberated iodine.

250. To 10 cc. of a dilute solution of morphine hydrochloride add 1 cc. of ammonia, 1 cc. of hydrogen peroxide (5-10 vols.), and 1 cc. of 1-4 per cent solution of copper sulphate ; a fine rose-red colour is produced (Denigès).

251. *Codeine*.—This alkaloid is the methyl ester of morphine. It does not contain the phenol group, consequently it neither gives the blue colour with ferric chloride, nor does it reduce iodic acid.

252. On the other hand, if 1-2 cc. of concentrated sulphuric acid and a single drop of dilute ferric chloride solution are added to a few drops of codeine solution, a marked blue colour is developed. The reaction is accelerated by heat and is also given by morphine.

253. *Atropine*.—A few crystals of atropine put in a porcelain capsule and moistened with a drop of concentrated nitric acid are dissolved without any development of colour. On evaporating to dryness upon the water-bath, and adding 2 or 3 drops of a freshly prepared solution of alcoholic potash, a violet colour appears (Vitali's reaction).

254. *Cocaine*.—Cocaine when heated, as atropine in the preceding paragraph (§ 253), gives no coloration, but gives off an odour resembling that of peppermint.

255. If not too dilute, solutions of cocaine are precipitated by potassium permanganate, which is itself, however, not reduced. Add to 1 cc. of a 2-3 per cent solution of

cocaine hydrochloride a few drops of 2 per cent permanganate solution; a clear bright violet precipitate results.

256. *Caffeine*.—See under purin compounds, § 354.

### Simplified Estimation of Quinine in Cinchona Bark

257. This method consists in freeing the alkaloid by ammonia and extracting the undried residue with chloroform. Into a flask of 125 cc. capacity put 10 grammes of finely powdered and sifted cinchona bark. The powder must be as fine as possible and sifted through a silk sieve (No. 80). Add 15 cc. of concentrated ammonia and 15-20 grammes of large shot; cork the flask, and shake well to make a homogeneous paste. After half an hour add 25 cc. of chloroform, cork, and shake again. The alkaloid is dissolved by the chloroform. The original cork is now replaced by one with a perforation, through which passes a short glass tube about 5-6 mm. in diameter and drawn out at its free end. The wider end is furnished with a small plug of absorbent cotton wool, which must not be packed too tightly. The flask is now inverted over another flask of 250 cc. capacity, when some of the clear chloroform extract escapes from the tube into the second flask. The first flask is now removed to allow of the entry of air and then re-inverted over the second, when a further small amount of chloroform extract is collected. By repeating this a few times the whole of the chloroform is easily separated off. A further quantity of chloroform is then introduced into the first flask and the extraction repeated; this is done four or five times.

The chloroform is then distilled off as far as possible on the water-bath, the flask being fitted to an ordinary condenser. To remove the last traces of chloroform a little air is bubbled through the flask, which is still kept warm.

The next procedure is the separation of the alkaloid from admixed resinous materials. For this purpose 10 cc. of 10 per cent sulphuric acid and 20 cc. of water are added to the residue in the flask, which is heated on the water-bath with frequent shaking. The alkaloids

are dissolved together with a small quantity of resin which has basic properties. To eliminate the latter, the mixture (which is still kept warm) is almost neutralized with dilute ammonia added drop by drop. When the liquid shows only a slight trace of acidity it is filtered, and the filter is washed through with boiling water. Finally, the filtrate and washings, collected in a porcelain evaporating basin, are neutralized *as accurately as possible* with dilute ammonia. As an indicator a strip of litmus-paper is used; after the neutralization is complete this should be well washed with boiling water, so as to lose none of the alkaloid. The neutralized liquid is then evaporated on the water-bath until a fine crystalline pellicle appears on the surface; it is then allowed to cool, and the sulphate of quinine collected on a round of filter-paper supported on a perforated porcelain plate about 2 cm. in diameter and drained by the aid of the filter-pump (see § 124). A little of the filtrate is used to wash any crystals remaining in the evaporating basin on to the filter. The residue on the filter is next washed with 2 cc. of distilled water added by means of a pipette. The filter-funnel is now inverted over a tared glass capsule, when the paper and precipitate (which should be in the form of a little cake) will fall into it. The paper is carefully removed and the residue dried to constant weight in the 100° air oven, and the final weight noted.

The quinine sulphate so obtained is only contaminated with a very small trace of cinchonidine; it is anhydrous, and, as will be seen from its formula  $(C_{20}H_{21}N_2O_2)_2H_2SO_4$ , contains 86.86 per cent of quinine.

### Estimation of Nicotine in Tobacco

258. This depends upon the extraction of the alkaloid by boiling with acidified water and precipitating the crude alkaloid as silicotungstate. This latter is then distilled with magnesia and the free nicotine so obtained is titrated alkalimetrically (G. Bertrand and M. Javillier). Take 12 grammes of tobacco, and put them in a flask with

four times their weight of .5 per cent hydrochloric acid. Bring to boiling, which is maintained gently for half an hour, taking care to condense the acid by a reflux condenser. If working with a half-litre flask a wide tube will answer the purpose. Cool the mixture under the tap, filter through cotton wool, and precipitate 250 cc. of the filtrate with silicotungstic acid, or potassium silicotungstate in 20 per cent solution. Collect the heavy precipitate, preferably by centrifuging, wash it with water slightly acidified with hydrochloric acid and containing also some of the precipitating reagent, then filter or centrifuge it again. Introduce the silicotungstate of nicotine so obtained into a flask with a long neck similar to that used for the estimation of acetic acid (§ 183) but of 250 cc. capacity. Add 2-3 grammes of calcined magnesia, suspended in a little water, and distil with a current of steam passing through the apparatus.

It is necessary to take care that the mixture does not become too dilute from condensation of the steam; there must be instead progressive concentration; this is effected by directly heating the flask containing the mixture, which should eventually be reduced to a few cubic centimetres in amount. A hundred cubic centimetres of water is enough to carry over 100-200 mgs. of the alkaloid, which is then estimated volumetrically. Standard sulphuric acid<sup>1</sup> is employed, such that 1 cc. corresponds to 10 mgs. of nicotine (3.024 grammes per litre). Alizarin is used as indicator; this turns from reddish-purple to yellow when the alkaloid is neutralized. The results obtained as above correspond to 10 grammes of tobacco.

<sup>1</sup> Decinormal acid can also be used. Nicotine ( $C_{10}H_{14}N_2$ ) has a molecular weight of 162; each cc. of N/10  $H_2SO_4$  corresponds to .0162 gramme of nicotine.





## CHAPTER XII

### THE PROTEINS AND THEIR ALLIES

259. THE proteins are complicated bodies of high molecular weight, which contain carbon, hydrogen, oxygen, and nitrogen; frequently sulphur and other elements are also present. Their essential characteristic is that they consist of a series of amino-acids linked together with the elimination of water. There are numerous varieties which have very different reactions; broadly, they are divisible into three classes<sup>1</sup>—the proteins proper, the proteids, and the proteoids.

The proteins proper are the most representative members of the whole group, and comprise such bodies as the albumins and globulins; when subjected to hydrolysis they break down into proteoses, peptones, and amino-acids. These last are obtained in varying amounts according to the protein examined, but all agree in containing the group  $-\text{CH.NH}_2-\text{COOH}$ , that is, they are  $\alpha$ -amino-acids (see p. 170).

The proteids may be regarded as resulting from the union of a protein molecule with another chemical group (phosphoproteids, metalloproteids, etc.); such a group is known as a prosthetic group.

The proteoids form a provisional group of bodies containing substances which, though allied in nature, cannot be included under the previous headings (ossein, keratin, gelatin, etc.).

<sup>1</sup> See note, p. 333.

### Precipitation Reactions of Protein Substances

260. *Precipitation by Precipitants of Alkaloids.*—Generally, aqueous solutions of protein bodies are precipitated by the reagents used for precipitating alkaloids. Such are mercuric chloride, potassium ferrocyanide, potassio-mercuric iodide, picric acid, tannic acid, phospho- and silico-tungstic acids, etc. With the majority of these reagents, and notably with potassium ferrocyanide, it is necessary to acidify, preferably with acetic acid.

261. *Precipitation by Metallic Salts.*—In addition to mercuric chloride, other salts of heavy metals, notably copper sulphate and lead acetate, are used as precipitants of proteins, with which they form insoluble compounds.<sup>1</sup>

262. *Precipitation by Neutral Salts.*—Protein solutions—other than those of peptones—are precipitated without the formation of definite chemical compounds by high concentrations of various neutral salts; more especially is this the case when the solutions are acidified with acetic acid. The most commonly used reagents are the sulphates of ammonium and magnesium and sometimes of zinc. These precipitates dissolve on dilution with water, and their formation is an entirely physical phenomenon, depending upon the abstraction of water by the salt and consequent appearance of the protein in solid form.

263. *Precipitation by Neutral Solvents.*—Certain bodies which are generally employed in organic chemistry as solvents, especially alcohol and acetone, are able to precipitate many of the proteins from their aqueous solutions. There are, however, exceptions to this rule, notably in the case of certain peptones, and some vegetable proteins, such as gliadin, which is soluble in dilute alcohol, but insoluble in water and absolute alcohol. In some cases the protein is not only precipitated but coagulated, and does not redissolve on dilution with water; coagulation is, however, not instantaneous, and is not complete without prolonged action of the precipitant.

<sup>1</sup> See note, p. 333.

Ether and chloroform have similar properties, but owing to their small solubility in water their action is much less marked.

264. *Coagulation by Inorganic Acids in the Cold.*—Proteins are coagulated in the cold by most inorganic acids; orthophosphoric acid is, however, an exception. Usually concentrated nitric acid is employed. A little of the acid is taken in a test-tube, and the protein solution is gently run on to its surface by means of a pipette, so as to form a supernatant layer. A trifle above the line of junction of the two layers an opaque zone is produced, owing to the precipitation of the protein matter. Excess of trichloroacetic acid (2-5 per cent) acts like the mineral acids.

265. *Coagulation by Heat.*—Solutions of albumins and globulins are coagulated by heat, at temperatures which vary with the particular kind of protein examined. This is not the case with certain "proteids" (see § 259), such as nuclein and casein, nor with the decomposition products of proteins such as albumoses and peptones. If a protein solution be acidified with one or two drops of acetic acid and boiled, the protein forms a flocculent precipitate if a sufficient quantity of neutral salts is present. If the fluid is too poor in these, about 1 per cent of sodium chloride should be added.

### Colour Reactions of Protein Substances

266. *The Xanthoproteic Reaction.*—To 5 cc. of protein solution (say 1 part egg-white to 9 parts of water) add .5 to 1 cc. of strong nitric acid. It generally forms a precipitate which partly redissolves on heating. Boil for about two minutes, and the liquid and precipitate become yellow; on the addition of ammonia the colour changes to orange. This reaction is due to the presence in the protein of a benzene nucleus, the nitro-substitution products of which have a yellow colour.

267. *Millon's Reaction.*—To 5 cc. of the protein solution prepared as above (§ 266) add 1-2 cc. of Millon's re-

agent,<sup>1</sup> and boil gently. A precipitate is formed which is at first pink and then changes from carmine to brick-red. If the heating is too prolonged it may decolorize and partially redissolve, hence the necessity for cautious heating at the beginning. This reaction is due to hydroxylated benzene groups, and is given strongly by ordinary phenol even in the cold. In proteins it is due to the presence of tyrosine (see pp. 170, 178), and as this substance is not ordinarily present in gelatin, Millon's reaction is not given by it.

268. *The Biuret Reaction*.—To a few cubic centimetres of protein solution add 1 cc. of 10 per cent caustic soda, so as to render it strongly alkaline, then add, drop by drop, a 1 per cent solution of copper sulphate. A rose-violet tint first appears, which on addition of more copper becomes purple. This colour, at least when it is obtained with biuret itself, is due to a compound in which the copper and the alkali both take part. It is given strongly by all the proteins, but it is especially marked with the proteoses and even more so with the peptones. With both these bodies the rose colour is particularly noticeable.<sup>2</sup> It seems that the presence of two  $-\text{CO}-\text{NH}-$  groups at least is necessary for this test to be given.

269. *Glyoxylic Acid Reaction* (Adamkiewicz, Hopkins, and Cole).—To 5 cc. of protein solution add 1-2 cc. of a solution of glyoxylic acid,<sup>3</sup> and mix thoroughly. Next by means of a pipette run 3-4 cc. of concentrated sulphuric acid to the bottom of the tube, so as to form a separate layer, and a fine violet colour will be developed at the line of junction of the two liquids. If the whole contents of the tube be

<sup>1</sup> *Preparation of Millon's Reagent*.—One part of mercury is dissolved in two parts of strong nitric acid, gentle heat being used towards the end if necessary. When solution is complete the liquid is diluted with twice its volume of water, shaken, allowed to stand, and the clear liquid poured off.

<sup>2</sup> For the use of ammonia as the alkali in performing the biuret reaction, see p. 334.

<sup>3</sup> A fresh solution of glyoxalic acid is easily made by adding to 10 cc. of a saturated watery solution of oxalic acid a fragment of sodium amalgam (containing 2 per cent of Na) the size of a pea. When evolution of hydrogen has ceased, the clear liquid is decanted.

now mixed, the liquid is coloured a clear violet throughout. This reaction is due to the presence of tryptophane in the molecule (see pp. 170, 179).

This reaction is inhibited by the presence of excess of chlorides and of nitrates, nitrites, and chlorates. It is given with great intensity by casein.

270. *Furfurol Reaction* (Liebermann).—Coagulate 5 cc. of the solution of egg-white (§ 266) by heat; decant the supernatant liquid as completely as possible, and treat the coagulum with 5 cc. of concentrated hydrochloric acid. Boil gently for three to five minutes, until the coagulum redissolves. A violet colour is produced which gradually changes to brown.<sup>1</sup> For this reaction a little of the protein which has been finely powdered may be used. The heating with hydrochloric acid produces furfurol, from certain carbohydrate groups in the molecule, and the furfurol so produced reacts with phenol nuclei to give the colour. Egg albumin gives the reaction very markedly.

271. *Sulphur Reaction*.—To 3 cc. of protein solution add an equal volume of strong caustic soda solution and 10 drops of a 10 per cent solution of neutral lead acetate. Boil, and in two or three minutes a black coloration, due to the formation of lead sulphide, appears. This reaction is due to the presence of cystine (see p. 170) in the protein molecule; the cystine is broken up by the hot alkali, and sodium sulphide is produced, which in turn reacts with the lead acetate to form black lead sulphide.

## ANIMAL PROTEINS

### Separation of Albumins and Globulins

272. Blood serum, egg-white, muscle-juice all contain solutions of albumins and globulins, coagulable by heat, and giving all the characteristic protein reactions just described. Globulins are distinguished from albumins by

<sup>1</sup> If the liquid is examined spectroscopically at this stage it shows an absorption band in the greenish-blue.

the fact that they are precipitated from their solutions by saturation with magnesium sulphate crystals; the same result is attained by the addition to the protein solution of its own volume of a saturated solution of ammonium sulphate, *i.e.* by half-saturation. On filtering, the albumins are found in the filtrate, from which they can be precipitated by saturation with crystals of ammonium sulphate.

273. *Preparation of the Proteins from Blood Serum.*—Horse or ox serum contains about 7-8 per cent of total proteins, globulin being present to about 4.5 per cent and albumin to 3.5 per cent. Take 100 cc. of such serum, add an equal volume of a saturated solution of ammonium sulphate, and stir thoroughly. The saturated ammonium sulphate solution is made by dissolving the salt in water at 50°-60° until no more enters into solution, then allowing to cool, and filtering. The addition of the ammonium sulphate solution causes a precipitate of serum globulin, which can be filtered off. Allow the filter to drain, and wash with a mixture of equal parts of water and the saturated ammonium sulphate solution, and press the precipitate between layers of filter-paper. The globulin, redissolved in the smallest possible quantity of warm water, can be isolated by dialysis or may be coagulated by heat. In the latter case the coagulated globulin can be collected on a filter, washed with boiling water, and dried. If now to the filtrate (from which the globulin has been precipitated by half saturation with ammonium sulphate) ammonium sulphate crystals are added to saturation, a further white precipitate is formed; this consists of serum albumin, which can be filtered off as before.

274. *Separation of Proteins from Egg-White.*—The white of fowls' eggs contains from 10 to 13 per cent of proteins. To isolate the globulin, collect the egg-white and divide it as much as possible with scissors; then add an equal volume of distilled water—this causes a certain amount of turbidity. The turbidity disappears on the addition of a few drops of sodium chloride solution; the liquid is then neutralized by adding a few drops of 10 per cent acetic acid, and strained

through a cloth. Collect the liquid in a graduated cylinder, and add an equal volume of saturated ammonium sulphate solution; shake the mixture and collect the resulting precipitate of ovoglobulin upon a filter.

The filtrate contains ovalbumin and ovomucoid; upon the addition of 10 per cent acetic acid the ovalbumin is precipitated and filtered off. Drain the filter, press the precipitate between filter-papers, and dissolve in a minimal quantity of warm water; on saturating this solution with crystals of ammonium sulphate, the ovalbumin is again precipitated. Collect it on the filter and wash with saturated ammonium sulphate solution.

### Crystallization of Albumin

275. *Crystallization of Serum Albumin.*—Serum albumin can, under certain conditions, give crystals, which apparently

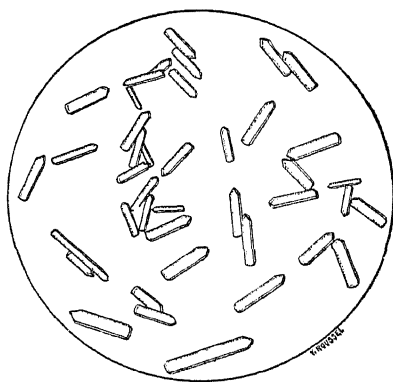


FIG. 28.—Crystals of Serum Albumin.

consist of combinations of the albumin with varying quantities of sulphuric acid. To prepare such crystals take 20 cc. of fresh horse serum and add an equal volume of saturated ammonium sulphate solution, so as to precipitate the globulin. To the clear filtered liquid ammonium sulphate solution is added, drop by drop, till a precipitate just begins



to appear, which redissolves on the addition of a few drops of water. While stirring add "normal" sulphuric acid, in such quantity that the turbidity first formed by its addition is redissolved; leave it in the cold, when little by little a precipitate appears, which after 12-24 hours shows characteristic boat-shaped crystals (Fig. 28). If the first addition of acid produces a voluminous precipitate instead of a mere turbidity, the precipitate should be redissolved by a little dilute ammonia, and "normal" sulphuric acid again added until the turbidity just appears.

276. *Crystallization of Egg Albumin*.—To the filtrate obtained after separation of the globulin (§ 274) add carefully, drop by drop, 10 per cent acetic acid until the precipitate produced just fails to dissolve on stirring; then add .1 cc. of the acid for every 10 cc. of filtrate. A copious precipitate forms, which next day will be found to be crystalline. It should be examined microscopically under a high magnification. If a few drops of the suspension of crystals are added to 1 per cent mercuric chloride solution, the crystals are rendered insoluble and preserve their form, so that they can be stained and kept as permanent preparations. It is necessary to work with perfectly fresh eggs in order to obtain a successful result.

### Preparation of Fibrinogen

277. Fibrinogen is a protein of the globulin type, which occurs in the blood plasma and is converted into fibrin upon coagulation. It is most easily obtained from horse serum, by bleeding the animal into a flask containing 100 cc. of 3 per cent sodium fluoride for every litre of blood to be treated. This stops coagulation; the red corpuscles may either be allowed to settle, or better separated by the centrifuge, when the plasma remains as a yellow supernatant fluid. Add to this an equal volume of a saturated solution of sodium chloride containing also .3 per cent of sodium fluoride. The fibrinogen then forms a gelatinous clotty precipitate. For its purification, collect upon a muslin strainer and

dissolve in the smallest possible quantity of a solution containing 1 per cent sodium chloride and .3 per cent sodium fluoride. The filtrate is reprecipitated by adding an equal volume of saturated sodium chloride solution. Repeat this procedure two or three times, and finally dissolve the precipitate by suspension in a little water; it enters into solution in consequence of the small amount of salt which adheres to it. This solution gives a flaky coagulum when heated to 55°-56°; on the other hand, it coagulates and contracts into a gelatinous clot on the addition of fibrin ferment (§ 423).

### VEGETABLE PROTEINS

278. Most vegetable proteins obtained from seeds resemble the animal globulins; generally speaking they are soluble in 10 per cent sodium chloride, and are precipitated either upon dilution or dialysis. They differ from the animal globulins in that they are usually not precipitated by magnesium sulphate nor by half saturation with ammonium sulphate; moreover, upon heating they either undergo very incomplete coagulation or do not coagulate at all. A certain number of vegetable proteins are distinguished by their solubility in 70-80 per cent alcohol either in the warm or at ordinary temperatures. These form a group by themselves.

#### Preparation of Edestin

279. Flax and hemp seeds contain, in addition to fatty matter, about 13 per cent of a globulin—edestin—insoluble in water but soluble in dilute saline solutions (Ritthausen, Osborne). To prepare edestin take 100 grammes of hemp seed and pound it as finely as possible in a mortar, put the mass into a glass tube closed at its lower extremity by a tap, above which is placed a tampon of absorbent cotton-wool. Introduce enough petroleum-ether to cover the powdered mass of seed, and allow to stand for half an hour. Then

open the tap to allow of the escape of the liquid, which is heavily charged with fat ; after closing the tap, extract again with a fresh supply of petroleum-ether. Repeat this five or six times, then shake out the fat-free residue on to a filter-paper and dry in the warm air oven at  $35^{\circ}$ . After drying re-grind the residue in a mortar. Take 50 grammes of this ground material and put it into a flask with 140 cc. of 10 per cent sodium chloride solution and 10 cc. of solution of baryta, saturated in the cold. Shake thoroughly and leave for two hours, shaking from time to time. Filter, and replace the first turbid portions of the filtrate on the filter ; the resulting liquid is slightly acid, of a yellowish-brown colour, and contains the edestin. The residue remaining on the filter is removed and ground up again with 50 cc. of 10 per cent sodium chloride. Refilter and add the filtrate so obtained to the previous one.

To separate the edestin, place the mixed filtrates in a parchment paper tube ("sausage-skin" dialyser), and dialyse against running water for three or four hours, when most of the sodium chloride will be removed and the edestin precipitated. Filter the contents of the dialyser, wash with distilled water, and dry at a low temperature.

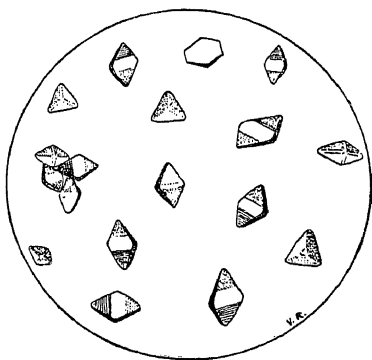


FIG. 29.—Crystals of Edestin.

280. *Crystallization of Edestin.*—To obtain edestin in a crystalline form take the material obtained as described in

the preceding paragraph, and add such an amount of 10 per cent sodium chloride solution as will give an approximately 8 per cent solution of edestin (about 80 cc. for the quantities mentioned in § 279). The solution is filtered, warmed to 60°, and has added to it twice its volume of water warmed to the same temperature. Let the liquid cool, and after a few hours in the refrigerator edestin is gradually deposited as crystals.

If it is simply desired to observe edestin crystals, grind 5 grammes of hemp seed in a mortar and macerate for half an hour on a water-bath at 60° with 25 to 30 cc. of 5 per cent sodium chloride solution; filter and allow to cool very slowly. Edestin will be deposited in characteristic octahedral crystals easily recognizable under the microscope (Fig. 29).

### Extraction of Gluten

#### *Separation of Gliadin and Glutenin*

281. Wheat flour consists essentially of starch (70 per cent) and of a nitrogenous material—gluten—which constitutes about 8-12 per cent. Gluten consists of a mixture of two proteins—gliadin, characterized by its solubility in alcohol; and glutenin, insoluble in cold water and alcohol, but soluble in very dilute solutions of alkalis.

282. *Extraction of Gluten.*—Mix 50 grammes of flour in a mortar with enough water to make a stiff paste and make it up into a ball. Rub the ball between the hands under a little stream of water, to wash away the starch which gradually disappears. When the escaping water is hardly turbid, break up the pasty mass and make it into several small balls, which are treated as before. The residue, consisting of moist gluten, is greyish, soft and elastic; it may be dried if desired.

283. *Preparation of Gliadin.*—The moist gluten obtained as above described (§ 282) is dried with filter-paper and broken into little pieces which are dropped into alcohol. The most convenient strength of alcohol to employ is 70 per cent;

thus for 10 grammes of moist gluten (which contain about 6-7 grammes of water, or two-thirds of the total weight) take 30 cc. of alcohol and 5 cc. of water. Warm the mixture upon a water-bath in a little flask fitted with a reflux condenser, shaking often and allowing it to digest in the warm alcohol for twenty-four hours. The alcohol is then decanted off, and replaced by more 70 per cent alcohol; the operation is repeated as long as any noticeable quantity of the residue is dissolved. The filtered mixed alcoholic extracts are concentrated *in vacuo*; finally a little more alcohol is added to dissolve any turbidity, and the liquid is poured in a fine stream into eight times its volume of cold water. The precipitated gliadin is collected, washed with water, and dried; gliadin constitutes 60-80 per cent of gluten.

284. *Preparation of Glutenin.*—The residue from the extraction with 70 per cent alcohol consists of glutenin; and in order to purify it, it is dried, powdered, and extracted with alcohol. The powder is then shaken with 30-50 cc. of potassium hydrate solution (.2 per cent). After complete solution it is filtered, neutralized with very dilute hydrochloric acid, and the precipitated glutenin collected, washed, and dried.

### Detection of Albumin in Urine

285. Boil 10 cc. of clear filtered urine in a test-tube; if it remains clear there is no albumin. A turbidity or flocculent precipitate may be due either to albumin or to mixed phosphates of earthy bases and ammonia. These last are soluble in the presence of dissolved carbon dioxide, but are precipitated when the gas is driven off by boiling. To distinguish between the two precipitates, add a drop of acetic acid, when the phosphates dissolve, and the albumin does not.

286. In some cases of urines of low specific gravity there are not enough salts present to ensure precipitation of albumin by boiling, or if a coagulum is produced it dissolves on the addition of acetic acid. It is then necessary to add

about 1 per cent of sodium chloride and to filter before boiling; and, indeed, this preliminary addition may be recommended as a routine procedure.

287. Urine may also be tested for the presence of albumin by the cold nitric acid test (§ 264). Some urines, rich in urates and not containing albumin, may give with nitric acid a cloudy zone suggestive of its presence. This source of error may be eliminated by diluting the urine before experiment with an equal volume of water. In this manner the precipitation of uric acid will not occur, and any turbidity will be due to albumin.

### Estimation of Albumin in Urine

288. This estimation is carried out on from 25 to 100 cc. of urine, according to the quantity of albumin present as roughly gauged by the qualitative tests. Add to the urine contained in a Bohemian glass beaker 1 per cent of sodium chloride and 1-4 drops of acetic acid, and heat on the water-bath till complete coagulation occurs; this is ensured by boiling the contents of the beaker for an instant. Allow the precipitate to settle, and decant the supernatant liquid over a folded filter; wash the precipitate thoroughly with boiling water, and transfer to the filter. Repeat the washings until the filtrate no longer gives a turbidity with silver nitrate solution.<sup>1</sup> By means of a jet from a wash-bottle carefully collect all the precipitate at the bottom of the filter; remove the filter-paper and place upon some more absorbent paper to take up excess of water. When the deposit and filter-paper are well drained, they can be easily separated by a spatula. Transfer the little block of albumin to a tared capsule and place it in the warm air-bath till a constant weight is obtained. The precipitate contains a small amount of saline matter which can be estimated after incineration. The result of this estimation is expressed as grammes of albumin per litre.

<sup>1</sup> The precipitate may also be separated and washed by using the centrifuge.

289. When the precipitate is very small, it may, instead of being weighed, be estimated by Kjeldahl's method, and multiplying the figure so obtained by the coefficient 6.25. It is not necessary to separate the deposit from the filter, but filter and residue may be directly treated with the sulphuric acid. For titration employ decinormal sulphuric acid.

# CHAPTER XIII

## PRODUCTS OF THE HYDROLYSIS OF PROTEINS

### Principal Products of the Decomposition of Proteins

#### (A) MONOAMINO MONOCARBOXYLIC ACIDS

1. Glycine. Amino acetic acid . . . . .  $\text{CH}_2(\text{NH}_2).\text{COOH}.$
2. Alanine.  $\alpha$ -amino propionic acid . . . . .  $\text{CH}_3.\text{CH}(\text{NH}_2).\text{COOH}.$
3. Valine.  $\alpha$ -amino isovaleric acid . . . . .  $\begin{array}{c} \text{CH}_3 \\ | \\ \text{CH}_3 \end{array} \text{CH}.\text{CH}(\text{NH}_2).\text{COOH}.$
4. Leucine.  $\alpha$ -amino isocaproic acid . . . . .  $\begin{array}{c} \text{CH}_3 \\ | \\ \text{CH}_3 \end{array} \text{CH}.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}.$
5. Isoleucine.  $\alpha$ -amino,  $\beta$ -methyl,  $\beta$ -ethyl propionic acid . . . . .  $\begin{array}{c} \text{CH}_3 \\ | \\ \text{C}_2\text{H}_5 \end{array} \text{CH}.\text{CH}(\text{NH}_2).\text{COOH}.$
6. Phenylalanine.  $\beta$ -phenyl,  $\alpha$ -amino propionic acid . . . . .  $\text{C}_6\text{H}_5.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}.$
7. Tyrosine.  $\beta$ -paraoxyphenyl,  $\alpha$ -amino propionic acid . . . . .  $\text{HO}.\text{C}_6\text{H}_4.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}.$
8. Serine.  $\beta$ -hydroxy,  $\alpha$ -amino propionic acid . . . . .  $\text{CH}_2.\text{OH}.\text{CH}(\text{NH}_2).\text{COOH}.$
9. Cystine di ( $\beta$ -thio,  $\alpha$ -amino propionic) acid . . . . .  $\text{HOOC}.\text{CH}(\text{NH}_2).\text{CH}_2.\text{S}—\text{S}.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}.$

#### (B) MONOAMINO DICARBOXYLIC ACIDS

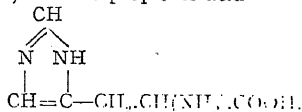
10. Aspartic acid,  $\alpha$ -amino succinic acid . . . . .  $\text{HOOC}.\text{CH}_2—\text{CH}(\text{NH}_2).\text{COOH}.$
11. Glutamic acid.  $\alpha$ -amino glutaric acid . . . . .  $\text{HOOC}.\text{CH}_2.\text{CH}_2—\text{CH}(\text{NH}_2).\text{COOH}.$

#### (C) DIAMINO MONOCARBOXYLIC ACIDS

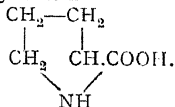
12. Arginine.  $\alpha$ -amino,  $\delta$ -guanidine valeric acid . . . . .  $\text{HN}=\text{C} \begin{array}{l} \diagup \text{NH}_2 \\ \diagdown \text{NH} \end{array} \text{CH}_2.\text{CH}_2.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}.$
13. Lysine.  $\alpha,\epsilon$ . diamino caproic acid . . . . .  $(\text{H}_2\text{N})\text{CH}_2.\text{CH}_2.\text{CH}_2.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}.$

#### (D) HETEROCYCLIC ACIDS

14. Histidine.  $\beta$ -imidazole,  $\alpha$ -amino propionic acid . . . . .

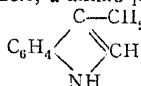


15. Proline.  $\alpha$ -pyrrolidine carboxylic acid . . . . .



16. Oxypoline . . . . .  $\text{C}_4\text{H}_7\text{N}.\text{OH}—\text{COOH}.$

17. Tryptophane.  $\beta$ -indole,  $\alpha$ -amino propionic acid . . . . .  $\text{C}—\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}.$





### Acid Hydrolysis of the Proteins of Serum

290. The soluble albumins and globulins are coagulated by mineral acids ; if heated the coagulum dissolves with the formation of acid-albumin ; this is next converted into albumoses and then into peptones ; finally, a mixture of mono- and poly-amino acids is produced. The formation of these disintegration products varies directly with the duration of the heating.

291. To study the progress of hydrolysis, introduce into a flask of 200 cc. capacity, 50 cc. of blood serum (ox or horse) and 6 cc. of concentrated sulphuric acid (corresponding to about 20 per cent by weight of the original serum taken). Close the flask with a cork through which passes a long glass tube to act as condenser, shake continuously, and take care that the froth does not reach to the condenser tube.

292. After ten minutes withdraw the flame, and remove 10 cc. of the liquid, adding 6 grammes of crystallized barium hydrate dissolved in a minimal quantity of boiling water (say 20 cc.). Ascertain that the liquid is slightly acid—adding a trifle more dilute acid if necessary—and filter. The filtrate gives a well-marked biuret reaction, and a sample saturated in the cold with solid ammonium sulphate gives a precipitate of albumoses. The formation of a blue precipitate, on the addition of a saturated solution of copper acetate to a fresh sample of the mixture, shows them to be primary albumoses.

293. The hydrolysis which has been interrupted after ten minutes from its commencement, is now restarted, and the heating continued for an hour. Then a further 10 cc. are removed and treated as before. The absence of a precipitate with ammonium sulphate and with copper acetate shows the absence of albumoses, while an intense biuret reaction indicates the presence of peptones.

294. The remainder of the liquid is allowed to boil for six to eight hours, when it will no longer give any biuret reaction as the peptones have all disappeared. A saturated boiling solution of barium hydrate is added till the liquid

becomes faintly alkaline; boil again, shaking all the time, and a smell of ammonia will be noticed. When the evolution of ammonia has stopped, add enough sulphuric acid, drop by drop, to produce a definite acidity. The filtered liquid is further acidified with 5 per cent sulphuric acid and 5 to 10 per cent phosphotungstic acid solution; this gives a precipitate of the diamino-acids. Filter off this precipitate, and add to the filtrate a solution of copper sulphate and enough caustic soda solution to render it slightly alkaline. By warming on the water-bath, a deep blue colour results, due to copper compounds of the mono-aminoacids.

### Reactions of Acid- and Alkali-Albumins

295. By acting upon proteins with acid or alkali, they are converted into certain ill-defined bodies, to which the names of "acid-" and "alkali-albumins" have been conventionally applied. They are insoluble in water and in solutions of neutral salts and are not coagulated by heat.

296. Acid-albumins remain in solution in the presence of excess of acid. On gradual addition of alkali they are precipitated when the neutral point is reached, but redissolve in excess of alkali.

297. Conversely, alkali-albumins dissolved in excess of alkali are precipitated upon neutralization with acid, and the precipitate redissolves in excess of acid.

### Characters of Albumoses and Peptones

298. Albumoses and peptones (with the exception of hetero-albumoses) are soluble in water and are not coagulated by heat. Hetero-albumoses differ in requiring small amounts of neutral salts for their solution. Albumoses are precipitated from a solution acidified with acetic acid by saturation with ammonium sulphate, while peptones are not. To examine these protein-disintegration products from a sample of commercial "peptone" proceed as follows: Dissolve

# BUMOSES AND PEPTONES

## REACTIONS OF PROTEINS, ALBUMOSES, AND PEPTONES

	Proteins.	Albumoses.		Peptones.
		Primary.	Secondary.	
Heat, in presence of dilute acetic acid . . . . .	Coagulation.	No coagulation.	No coagulation.	No coagulation.
Ammonium sulphate . . . . .	Precipitate in neutral solutions.	{ Precipitate with half saturation in presence of acetic acid. }	Precipitate with saturation in presence of acetic acid. }	No precipitate.
Nitric acid . . . . .	Coagulation.	{ Precipitate soluble on warming, reappearing in cold. }	No precipitate (unless in medium saturated with NaCl).	{ No precipitate (even in medium saturated with NaCl). }
Potassium ferrocyanide and acetic acid . . . . .	White precipitate.	{ Precipitate soluble on warming re-appearing in cold. }	No precipitate.	No precipitate.
Copper acetate . . . . .	Coagulation.	Blue precipitate.	No precipitate.	No precipitate.
Mercuric chloride (sat. sol.) .	Coagulation.	White precipitate.	White precipitate.	Partly precipitated.
Lead acetate . . . . .	Coagulation.	White precipitate.	White precipitate.	Partly precipitated.
Picric acid (sat. solution) .	Yellow precipitate.	Yellow precipitate.	Yellow precipitate.	Yellow precipitate (in conc. solutions).
Phosphotungstic acid in presence of sulphuric acid /	White precipitate.	White precipitate.	White precipitate.	White precipitate.

5-10 grammes in 50 cc. of water, and acidulate with 2 drops of acetic acid; add powdered ammonium sulphate and shake to assist its solution. When there is a small excess of the undissolved salt, saturation is complete, and almost all the albumose is precipitated. Filter, and the peptones present in the filtrate will give a strong biuret reaction; the solution can be used to study the reactions of peptones.

299. The precipitate left on the filter is washed with a saturated solution of ammonium sulphate, then drained and dried between layers of filter-paper. On dissolving in a little water, the albumose reactions can be studied according to the scheme shown in the table.

### General Characters of Amino-Acids

300. The  $\alpha$ -amino-acids resulting from the hydrolysis of proteins are solid bodies, more or less soluble in water; some, such as glycocoll and alanine, have a sweet taste.

301. Heated on the water-bath with a little water, and hydrate or carbonate of copper, these latter are dissolved, forming dark blue salts of the amino-acids.

302. A watery solution of amino-acids, rendered neutral to phenolphthalein, becomes strongly acid on the addition of neutral formaldehyde.

### Preparation of Glycocoll (or Glycine)

303. This is most easily obtained by the hydrolysis of gelatine or silk. Heat in a flask, fitted with a reflux condenser, 20 grammes of gelatine with 60 cc. of concentrated hydrochloric acid, until hydrolysis is complete; this generally requires seven or eight hours. After this time, take out 2 cc. of the liquid, add 5-10 cc. of a dilute solution of silver nitrate, and filter. The almost colourless filtrate should give no biuret reaction. When this point is reached, turn the liquid out of the flask into an evaporating basin,

and evaporate to a syrup on the water-bath. Take up the warm residue with 50-60 cc. of absolute alcohol, and pass dry hydrochloric acid gas through it, to saturation, shaking well during the operation so as to ensure solution. When saturation is complete, heat for a few seconds to boiling, and then cool to  $0^{\circ}$  by putting it on ice. After twenty-four hours, crystals of glycocoll-ester hydrochloride are formed, which are collected on a filter and washed with a little cold alcohol. When dried, the crystals melt at  $144^{\circ}$ . To obtain pure glycocoll, the crystals are dissolved in a little water and heated with a slight excess of precipitated lead hydrate. The mixture is now evaporated to dryness, and extracted with cold water, which dissolves the glycocoll. Filter, and accurately precipitate the lead by addition of dilute sulphuric acid, drop by drop; refilter and concentrate until crystallization occurs.

### Characters of Glycocoll (or Glycine)

304. Glycocoll forms colourless crystals of sweetish taste soluble in water and precipitated by alcohol when not too dilute. The aqueous solution, on the addition of ferric chloride, yields a red colour similar to that produced by formates and acetates.

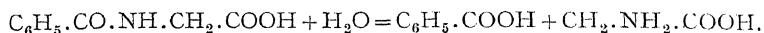
305. On gently heating a solution of glycocoll with copper hydrate, the latter is dissolved, forming a dark blue solution. The blue solution, when filtered and concentrated, gives a crop of needle-shaped blue crystals of copper glycocollate  $(\text{CH}_2.\text{NH}_2.\text{COO})_2\text{Cu} + \text{H}_2\text{O}$ ; these on incineration yield 34.67 per cent of copper oxide.

306. Watery solutions of glycocoll on the addition of phenol and sodium hypochlorite give a deep blue colour. The reaction is analogous to that given by ammonia under the same conditions (§ 531), but is even more delicate. In order to perform this test, add to 5 cc. of glycocoll solution 1 cc. of phenol solution (3-4 per cent), mix and add one or two drops of sodium hypochlorite solution; a blue colour then gradually develops.

### Characters of Hippuric Acid

307. Hippuric acid or benzoyl glycocoll crystallizes in long brilliant needles, soluble in 600 parts of cold water, but readily soluble in hot water. It melts at  $187^{\circ}$ – $188^{\circ}$ , and gives, if sufficiently heated, a sublimate of benzoic acid; at the same time is given off an aromatic odour accompanied by that of hydrocyanic acid.

308. Upon hydrolysis it yields benzoic acid and glycocoll.



To demonstrate the formation of benzoic acid, heat 1 gramme of hippuric acid with 10 cc. of 10 per cent hydrochloric acid to boiling for twenty minutes. On cooling, benzoic acid crystallizes out in long needles. Benzoic acid can readily be extracted from the solution by ether; its melting-point is  $121.5^{\circ}$ .

### Characters of Leucine

309. A small amount of leucine heated in a dry test-tube partially sublimes on the walls of the tube. On further heating, white fumes are produced, and a characteristic odour of amylamine is given off.

### Characters of Aspartic Acid

310. This acid is produced by the acid hydrolysis of asparagine (§ 323). It gives with lead salts a very slightly soluble white precipitate which crystallizes in needles. On addition of a saturated solution of copper acetate, solutions of aspartic acid give a sky-blue precipitate of copper aspartate ( $\text{C}_4\text{H}_5\cdot\text{CuNO}_4 + 5\text{H}_2\text{O}$ ), which crystallizes in needles.

### Preparation of Glutamic Acid

311. Glutamic acid is produced in abundance by hydrolysis of the vegetable proteins which constitute gluten. For

its preparation, boil for about eight hours 20 grammes of dried gluten with 100 cc. of concentrated hydrochloric acid in a flask fitted with a reflux condenser. The upper end of the condenser is closed by a cork through which passes a bent glass tube, leading into a flask containing water, the free end of the tube ending about 1 to 2 centimetres above the surface of the water. The object of this addition to the condensing system is to absorb vapours of hydrochloric acid gas which are disengaged during the boiling. When hydrolysis is complete, cool the hydrolysed mixture, and maintain at  $0^{\circ}$  by means of ice; then pass a current of dry hydrochloric acid gas to saturation. Leave then, for twenty-four hours, and an abundant crop of crystals of the hydrochloride of glutamic acid will result; this is only very slightly soluble in concentrated hydrochloric acid (Hlasiwetz and Habermann).

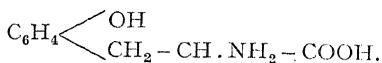
312. If it is desired to isolate the acid, proceed as follows. The crystals are removed and drained, washed with a little hydrochloric acid, and dissolved in a small quantity of water. Milk of lime is added in slight excess, the whole evaporated to dryness on the water-bath, and extracted with boiling alcohol. The alcohol dissolves the calcium chloride present. After having extracted thoroughly with alcohol, the residue is dissolved in a little boiling water, and a solution of oxalic acid cautiously added to precipitate calcium oxalate. The liquid is filtered and concentrated by evaporation, when glutamic acid crystallizes out (P. Thomas).

### Characters of Phenylalanine

313. Phenylalanine is easily transformed by oxidation into phenylacetic aldehyde,  $C_6H_5 \cdot CH_2 \cdot CHO$ , which has an odour of cinnamon and is reminiscent of the smell of Balsam of Tolu. To demonstrate this property of phenylalanine, take a little in a test-tube with 2 cc. of water, add 10 drops of concentrated sulphuric acid and a crystal of potassium bichromate. The liquid becomes brown and the characteristic odour is evolved (E. Fischer).

### Characters of Tyrosine

314. Tyrosine ( $\beta$ -paraoxyphenyl  $\alpha$ -amino propionic acid) combines the properties of an  $\alpha$ -amino acid and of a phenol.



It crystallizes in fine white needles grouped in sheaves or rosettes. In order to see the characteristic crystals, take an ammoniacal solution of tyrosine, allow it to evaporate spontaneously upon a glass slide, and examine microscopically (Fig. 30); do not omit to verify their solubility in concentrated hydrochloric acid and ammonia.

315. *Millon's Reaction*.—Tyrosine is but very slightly soluble in water (about .5 gramme per litre); in spite of this, 1 cc. of an aqueous solution of tyrosine gives a strong Millon reaction. The heating must be very slight; on too strong heating the red colour disappears and gives rise to a yellow tint.

316. *Tyrosinase*.—In the presence of this enzyme tyrosine undergoes rapid oxidation when exposed to the air. To

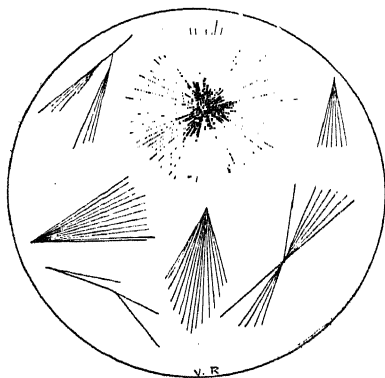


FIG. 30.—Tyrosine Crystals.

5 cc. of a saturated watery solution of tyrosine add 3-5 drops of a glycerol extract of fungi rich in tyrosinase (see § 439); a red colour soon appears (5-15 minutes), which becomes blackish-brown after a few hours (G. Bertrand).



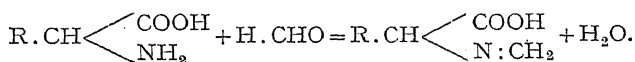
317. *Piria's Test*.—Dissolve a little tyrosine in a drop or two of concentrated sulphuric acid, at the bottom of a test-tube, and warm on the water-bath for ten minutes. Dilute with 2-3 cc. of water, neutralize the acid by boiling with a pinch of barium carbonate, and filter. The colourless, clear solution of barium tyrosine-sulphonate, on the addition of a drop of dilute ferric chloride solution, gives a fine violet colour.

### Reactions of Tryptophane

318. Tryptophane is liberated during the tryptic digestion of proteins. Free or combined it gives the glyoxylic reaction (§ 269) strongly. When it is free, it gives the following reaction. On addition of bromine water, drop by drop, a red-violet colour appears, then a precipitate like wine dregs. The colour is decidedly evanescent.

### Titration of Amino-Acids

319. Amino-acids resulting from protein hydrolysis contain the characteristic group  $-\text{CH}.\text{NH}_2-\text{COOH}$ . They act like acids towards strong bases, and like bases towards strong acids. On treatment with formaldehyde, the  $\text{NH}_2$  group is eliminated, and a methylene derivative produced, which is acid in reaction and can be titrated with phenolphthalein as indicator.



This reaction is made use of in the titration of amino-acids (Sørensen).

320. To estimate glycocoll, for example, measure accurately 10 cc. of the glycocoll solution, add two or three drops phenolphthalein, and neutralize. Then add 20 cc. of a mixture of equal parts commercial "formalin" (40 per cent formaldehyde) and water (previously carefully neutralized by soda, added drop by drop, using phenolphthalein

as indicator). On addition of the formaldehyde solution a strong acid reaction is produced; the mixture is then titrated with decinormal soda till a permanent pink is obtained. Each cc. of decinormal soda corresponds to  $\cdot 0075$  gramme of glycocoll.

With the amino-acids the quantities corresponding to 1 cc. of decinormal soda vary with their molecular weights. Thus :

$\cdot 0075$	gramme	Glycocoll.
$\cdot 0089$	„	Alanine.
$\cdot 0131$	„	Leucine.
$\cdot 0165$	„	Phenylalanine.
$\cdot 0181$	„	Tyrosine.

All correspond to 1 cc. of  $N/10$  NaOH.

321. When estimating amino-acids in complex mixtures still containing proteins, or in turbid fluids resulting from a digestion, a larger quantity of indicator must be used, and the titration is a little less accurate. Should the liquid be too highly coloured, the tint will be difficult to see, and so the colouring matter should be removed as far as possible by adding barium chloride and silver nitrate solutions (the barium chloride being in slight excess). The resulting precipitate of silver chloride will carry down with it most of the colouring matter. For example, take 25 cc. of the coloured liquid under examination, add 10-12 cc. of 10 per cent solution of silver nitrate, and the same quantity of 10 per cent barium chloride, and make up to 50 cc. in a graduated flask, and filter. Make the titration upon an accurately measured quantity of the filtrate.

## CHAPTER XIV

### AMIDES

#### Characters of Asparagine

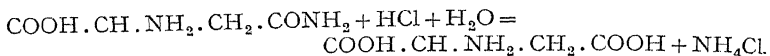
322. ASPARAGINE is widely distributed in the vegetable kingdom, especially in rapidly growing parts of plants (buds and shoots) ; it is the amide of aspartic acid and crystallizes in fine colourless transparent crystals, slightly soluble in cold water (about 2 per cent) but more soluble with heat. Solutions of asparagine, on the addition of a saturated solution of copper acetate, give a sky-blue crystalline precipitate, which may take some time to appear ; its development is favoured by shaking.

#### Hydrolysis of Asparagine and Formation of Copper Aspartate

323. Put 10 cc. of a 2 per cent solution of asparagine into a test-tube with three pellets of potassium hydrate, heat on a boiling water-bath from twenty to thirty minutes. Ammonia is given off and can be recognized by its smell and action on red litmus-paper. At the end of this time exactly neutralize with glacial acetic acid, and add 1-2 cc. of a 10 per cent solution of copper acetate. On cooling, a fine blue crystalline precipitate of copper aspartate is produced ; this is only slightly soluble in water ; its formation may be hastened by shaking and rubbing with a glass rod.

### Titration of Asparagine

324. Keep for an hour upon a boiling water-bath a mixture of 20 cc. of the asparagine solution with 2 cc. of concentrated hydrochloric acid. The asparagine is converted into aspartic acid, half of its nitrogen being converted into ammonium chloride, thus

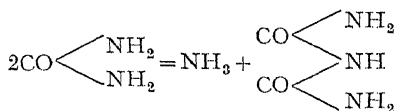


Turn the mixture into the flask of an Aubin's apparatus, add the washings of the flask in which the hydrolysis was carried out, and make up the total volume to 200 cc. with water. Finally, add 10 cc. of caustic soda solution. Boil, and titrate the ammoniacal distillate with N/5 sulphuric acid, using methyl orange as indicator. Each cubic centimetre of acid used corresponds to 3.4 mgs. of ammonia, or to .03 gramme of crystallized asparagine ( $\text{COOH} - \text{CH} \cdot \text{NH}_2 - \text{CH}_2 - \text{CONH}_2 + \text{H}_2\text{O}$ ). This method of titration is only available in the absence of any other bodies which split off ammonia on boiling with hydrochloric acid.

### Characters of Urea

325. *Formation of Urea Nitrate*.—Evaporate 5 cc. of urine upon a water-bath till the volume is reduced to about 1 cc. (*not* to dryness). Cool, and add 1 cc. of nitric acid; a white crystalline mass of urea nitrate separates immediately; microscopically this is seen to consist of transparent plates.

326. *Production of Biuret*.—Heat a small quantity (.1 gramme) of urea in a test-tube till it melts, and keep it at about the same temperature for a second or so. Ammonia is given off, and the mass solidifies, the urea being converted into biuret.

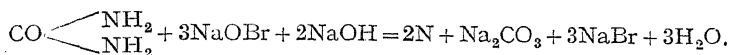


Allow to cool, and take up the residue in 2 cc. of water

warm slightly to assist solution, and add 1 cc. of 10 per cent caustic soda ; then add drop by drop a 1 per cent solution of copper sulphate ; the first drops produce a red-violet colour, which becomes bluer on further addition of copper.

### Estimation of Urea in the Urine

327. *Sodium Hypobromite Method.*—This method is based upon the decomposition of urea into carbon dioxide, free nitrogen, and water by sodium hypobromite :



In an alkaline medium free nitrogen only is evolved (the carbon dioxide being absorbed by the excess of alkali), its volume is measured, and from this the quantity of urea present is calculated. It must be remembered that by this method only 92 per cent of the nitrogen present is measured if sugar is not present ; but in the presence of glucose or saccharose, all the nitrogen is given off. Hence the operation should either be performed in the presence of such sugar, or, if this is not done, the nitrogen figure obtained must be multiplied by  $\frac{100}{92}$  ; this correction is unnecessary in the case of markedly diabetic urines. A further point to be remembered is that the hypobromite also decomposes other nitrogenous bodies in the urine, such as ammonium salts, creatinine, etc. Hence a higher nitrogen content is obtained than would be accounted for by the urea alone. In ordinary clinical determinations this source of error is small and is commonly ignored.

328. The apparatus employed consists of a glass tube closed at one end, of about 35 cc. capacity, and furnished with a bulb near the open end ; the tube is graduated in cubic centimetres and tenths of a cc. (G. Bertrand). Commence operations by moistening the inside of the tube ; run in by means of a pipette 10 cc. of hypobromite solution,<sup>1</sup> then

<sup>1</sup> The hypobromite solution is prepared by dissolving 1 cc. of bromine in 7 cc. of strong soda solution (36° B.), which has previously been diluted by the addition of 25 cc. of water.

add gently and without mixing with the hypobromite either 5 cc. of soda solution (4 per cent), or of 10 per cent sugar

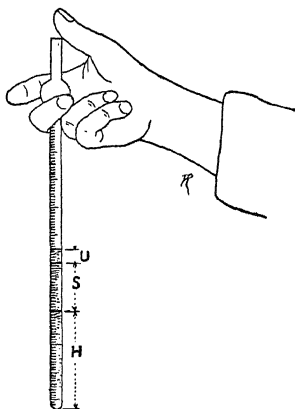


FIG. 31.—Bertrand's Ureometer.

solution (see § 327); finally, add 1 cc. of urine accurately measured, so that it forms a layer above the preceding two.

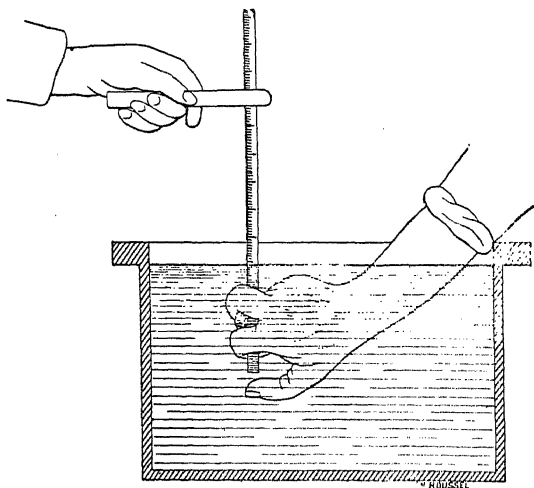


FIG. 32.—Method of holding Ureometer.

Read off the total volume in the ureometer which the liquid occupies; firmly close the open end with the thumb, and

invert the tube three or four times to ensure thorough mixture of the contents. When the evolution of gas has ceased, plunge the end of the apparatus (the mouth being still closed by the operator's thumb) beneath the surface of a water trough, remove the thumb, and allow the excess of hypobromite to escape. Completely submerge the tube in the water, the temperature of which should be near that of the laboratory. Next, holding the tube vertically by means of a wooden clip, adjust it so that the level of the liquid inside the tube corresponds with that of the water in the trough. The contained gas is at the atmospheric pressure  $H$  minus the pressure of aqueous vapour  $F$  at the temperature  $t$  (since it is saturated with water vapour at this temperature); the values of the pressures of aqueous vapour at different temperatures are given in the table (p. 329).

Close the mouth of the apparatus once more with the thumb, remove from the water, and read off the volume occupied by liquid. The difference between this volume and the volume occupied by liquid before the experiment gives the volume of nitrogen measured at  $t^\circ$  and at pressure  $H - F$ . To reduce this figure to that obtaining at  $0^\circ$  and 760 mm., the following formula is used :

$$V_0 = \frac{V_t}{1 + 0.00367t} \times \frac{H - F}{760}.$$

The volume  $V_0$ , expressed in cubic centimetres and multiplied by 1.2511 (=weight of 1 litre of N at  $0^\circ$  and 760 mm.), gives in milligrammes the weight of nitrogen obtained. This weight must be multiplied by  $\frac{100}{92}$  if sugar was not present during the experiment. The calculation is simplified by the table (p. 187). From the equation representing the reaction (§ 327) it is seen that 28 grammes of nitrogen correspond to 60 grammes of urea; hence by multiplying the weight of nitrogen as obtained above by  $\frac{60}{28}$ , the number of milligrammes of urea in 1 cc. of urine can be found; the figure resulting is identical with that expressing grammes per litre.

In order to expedite calculation, it is usual in practice

to multiply the volume of nitrogen (reduced for  $0^{\circ}$  and  $760^{\circ}$  mm.) by the factor 2.91 (or 2.68 if sugar is present). These coefficients can easily be deduced from the foregoing calculations.

329. *Calculation of Urea by Formation of Ammonia.* (*This method is inapplicable to urines containing sugar.*)—For exact determinations the hypobromite method is inapplicable. The best procedure depends upon the transformation of urea into ammonia by heating with a concentrated solution of magnesium chloride, which, unlike hypobromite, reacts neither with uric acid, hippuric acid, nor with creatinine (Folin). On deducting the amount of ammonia originally present, we have all the data necessary for calculating the urea.

330. Measure exactly 5 cc. of urine into a conical flask ; add 20 grammes of crystallized magnesium chloride and 5 cc. of concentrated hydrochloric acid. To the mouth of the flask fit a perforated rubber cork transmitting a glass tube 20-25 cm. long and 8-10 mm. in diameter, which is cut off obliquely at the lower end ; this serves as a condenser. On heating, the melted mass soon boils, and as the drops of condensed liquid fall back into the flask a distinct crackling sound is noticed. Keep slowly boiling for an hour, and add from time to time down the condensing tube one or two drops of concentrated hydrochloric acid, to compensate for loss by evaporation. It is a good plan to add at the same time a drop of helianthine as an indicator, and so to ensure acidity. Any alkalinity would of course lead to a loss of ammonia.

When the hydrolysis is finished, turn the contents of the flask into the flask of Aubin's ammonia apparatus, repeatedly washing out with water and making up the total volume to about 250 cc. Then add 5 cc. of pure soda solution ( $36^{\circ}$  B.), and distil till evolution of ammonia ceases. Titrate with N/5 sulphuric acid, as in § 40. As the soda solution and magnesium chloride may not be quite ammonia-free, a control experiment is desirable, and the correction necessary for such possible impurity of the reagents can be made. From the result the amount of ammonia normally present in urine,



and determined according to § 537, must be subtracted. By multiplying the final result, expressed as ammonia, by the factor  $\frac{6}{3.4}$  ( $=1.7647$ ), the corresponding amount of urea is found.

### Relation of Nitrogen Volume to Weight

If  $V_t$  be the volume of nitrogen measured at pressure  $H$  and at the temperature  $t^\circ$  of the water trough, the weight of this nitrogen is given by the formula

$$W = V_t(H - F)n$$

when  $n$  has the value  $\frac{.0012511}{760(1 + .00367t)}$  grammes.

TABLE OF VALUES OF  $n$  FROM  $10^\circ$  TO  $30^\circ$

Temperature.		Temperature.	
10° . . .	.0015879	21° . . .	.0015284
11° . . .	.0015824	22° . . .	.0015232
12° . . .	.0015767	23° . . .	.0015180
13° . . .	.0015712	24° . . .	.0015129
14° . . .	.0015658	25° . . .	.0015079
15° . . .	.0015603	26° . . .	.0015027
16° . . .	.0015549	27° . . .	.0014997
17° . . .	.0015495	28° . . .	.0014927
18° . . .	.0015442	29° . . .	.0014878
19° . . .	.0015388	30° . . .	.0014829
20° . . .	.0015336		

## CHAPTER XV

### “PROTEIDS” AND THEIR DERIVATIVES

#### General Remarks on “Proteids”

331. “PROTEIDS” are by our definition compounds of a protein with prosthetic groups of varying nature; their most representative members may be classified as follows, according to the nature of the prosthetic group.

(1) *Glucoproteids*, in which the prosthetic group is a nitrogen containing carbohydrate derivative, most usually glucosamine.

(2) *Phosphoproteids*, formed by the combination of a protein molecule with a phosphorized group.

(3) *Metalloproteids*, compounds of a protein and of a complex containing sometimes iron, copper, manganese, vanadium, etc. The properties vary according to the different groups.

#### Glucoproteids

332. The glucoproteids are soluble in water, and their solutions are not coagulated by heat. They behave as weak acids, and dissolve in alkalis to viscous solutions which generally precipitate on addition of acids. Heated for some hours with dilute mineral acids, they undergo hydrolysis with the liberation of glucosamine, and the liquid reduces alkaline copper solutions.

333. On heating a glucoproteid solution with a little soda for a few seconds and adding a 2 per cent solution of para-dimethylamino-benzaldehyde in 10 per cent hydrochloric

acid till the mixture just becomes acid, a red-violet colour is produced which is accentuated by heat. Of this class of proteids there are two groups: the mucins, insoluble in water and precipitated from their natural state or from alkaline solutions by acetic acid; and the mucoids, soluble in water and not precipitated by acids.

### Preparation of Mucin

334. Mucin occurs in a large number of the glands in the respiratory and digestive tracts; it is easily extracted from the salivary glands. Macerate a well-pounded sub-maxillary gland (of the ox, *e.g.*) in 20 parts of .1 per cent soda; shake frequently, and after half an hour strain through muslin and then filter through Chardin's paper. Precipitate the filtrate by the addition of just enough acetic acid, added drop by drop, shaking meanwhile. The precipitate collected on a filter and well washed gives the xanthoproteic, Millon, biuret and glyoxylic acid reactions, as well as the reaction with *p*-dimethylamino-benzaldehyde (§ 333). It is soluble in dilute soda or hydrochloric acid.

Boiled with 6-8 per cent hydrochloric acid for a couple of hours it reduces Fehling's solution (hydrochlorate of glucosamine).

### Preparation of Ovomucoid

335. Egg-white contains, in addition to albumin and globulin, a small quantity of non-coagulable proteid belonging to the glucoproteid group; to this the name of "ovomucoid" is given, and it differs from the mucins inasmuch as it is not, like them, precipitable by acids. For its preparation, carefully break two eggs, and having separated the yolks dilute the whites with four times their volume of water. Acidify with acetic acid, and boil, shaking meanwhile so as to assist the coagulation of the proteins. When the liquid becomes clear, filter and collect the filtrate, which contains ovomucoid. Next concentrate the filtrate to half its bulk on a water-bath, and saturate the still hot liquid with solid

ammonium sulphate, which precipitates the ovomucoid, and filter off the boiling fluid. Concentrated solutions of ovomucoid can also be precipitated by the addition of four to five times their volume of alcohol. The precipitate gives the xanthoproteic, Millon, biuret and *p*-dimethylamino-benzaldehyde reactions.

336. *Glucosamine*.—Dissolve the precipitate obtained as above (§ 335) in 25 cc. of 10 per cent hydrochloric acid; put the mixture in a flask fitted with a long tube to act as condenser, and boil gently for three hours. The liquid will then be found to have acquired reducing properties; neutralize and filter, then estimate the reducing power of 5 cc., which can be expressed in terms of glucose. For each gramme of sugar thus calculated add 10 cc. of benzoyl chloride and 100 cc. of 10 per cent soda; shake vigorously, and a white precipitate of benzoyl-glucosamine forms; this may be collected on a filter, washed, and dried.

### Phosphoproteids

337. The phosphoproteids owe their acid properties to a phosphorized group; they are soluble in dilute alkalis, and are precipitated from these solutions by dilute acids. They may be divided into two groups:

- (1) Para- or pseudo-nucleoproteids, which do *not* contain purine bases united to phosphoric acid in their molecule.
- (2) True nucleoproteids; which are decomposable with the liberation of nucleic acid.

338. The most prominent members of the first group are the casein of milk and vitelline of egg-yolk. Treated with 10 per cent caustic soda for twenty-four to forty-eight hours at 37°, all their phosphorus is split off as phosphoric acid (Plimmer and Scott).

### Preparation of Casein

339. Measure out 50 cc. of milk and dilute with 150 cc. of water; add drop by drop (shaking all the time) 1 per

cent acetic acid so that a precipitate forms, but avoid excess of acid. For a litre of milk about .75 gramme of acetic acid ( $\text{CH}_3\text{COOH}$ ) are required; for 50 cc., as taken above, 3-5 cc. of 1 per cent acetic acid are needed. Allow the mixture to stand, filter, remove the precipitate from the filter, and rub up with a few drops of ammonia, adding 20 cc. of water to make a solution. Filter on a moistened filter, repeating the operation several times if necessary. Add 1 per cent acetic acid to the clear or opalescent solution, and collect the resulting precipitate of casein. This may be dehydrated by successive washings of alcohol and ether, and then dried.

### Preparation of Ovovitelline

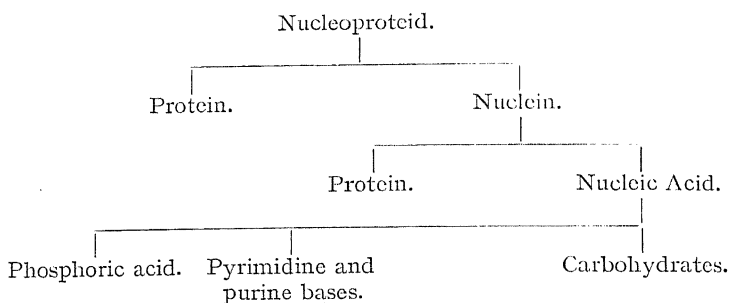
340. Take the yolks of two fresh eggs, freed as completely as possible from the white, shake them thoroughly with an equal volume of 10 per cent sodium chloride. Turn the mixture into a flask, adding 50 cc. of ether, shake up thoroughly, and allow to stand for twenty-four hours. The ether is then poured off, and replaced by a fresh quantity, and the whole treated as before. After a third extraction the saline residue is diluted with twenty times its volume of water, the resulting precipitate carefully collected and washed several times by decantation. The impure ovovitelline may be re-dissolved in 10 per cent sodium chloride, once more extracted with ether, and re-precipitated by addition of excess of water; the precipitate is treated by successive washings of alcohol and ether, and dried.

### Nucleoproteids

341. These proteids have an acid character, and contain nucleic acids wherein the phosphorus combined as phosphoric acid is united with purine and pyrimidine bases and a carbohydrate group of the pentose variety. Nucleoproteids are soluble in water, in dilute alkalis, and in weak solutions of neutral salts; they are precipitated from these solutions by dilute acids, but are soluble in excess of acid.

342. The majority of nucleoproteids are not coagulated by heat, and can be extracted from pounded organs containing them by boiling water. Dilute solutions of alkalis are also used for their extraction (ammonia  $\cdot 1\cdot 2$  per cent, sodium carbonate or bicarbonate  $\cdot 25\cdot 5$  per cent); from these solutions they may be precipitated by dilute acetic acid.

343. Upon hydrolysis, nucleoproteids give the following series of decomposition products :



The relative richness in phosphorus increases from 1-3 per cent for nucleoproteids to 4-7 per cent in the nucleins, and 9-10 per cent in the nucleic-acids.

### Extraction of Ferratin from the Liver

344. A nucleoproteid occurs in the liver, which like most other bodies of this group contains iron, but in much larger amount. To extract it, mince in the mincing-machine 50 grammes of pig's liver, add 200 cc. of water, boil for five minutes, and filter. The opalescent filtrate is acidified with a 10-15 per cent solution of tartaric acid until a precipitate is formed. Collect the precipitate by centrifuging or filtering, and wash it with a little water. This precipitate consists of ferratin. Dissolved in a few drops of dilute ammonia, it gives no precipitate on addition of ammonium sulphate. On incineration only a small amount of ash remains, in which iron can easily be detected (§ 23); the metal occurs

in an organic combination. The ashes are equally rich in phosphoric acid, which can be demonstrated as shown in § 14.

### Preparation of Nucleic Acid from Yeast

345. Take about 40 grammes of commercial compressed yeast and add 30 cc. of soda solution ( $36^{\circ}$  B.). Break up the mass thoroughly and leave for a quarter of an hour; then add 20 cc. of water, shake well and add at the same time 10-20 cc. of a 10 per cent solution of ferric chloride, which will produce a gelatinous precipitate. The mass, which should be almost homogeneous, is drained upon a cloth placed in a funnel, so that the almost clear liquid can be collected in a beaker. The residue is washed with 50 cc. of warm water (at  $60^{\circ}$ - $70^{\circ}$ ) and again drained on the cloth. The brownish filtered liquid is added to an equal volume of alcohol, containing just enough hydrochloric acid to render it slightly acid. A precipitate of nucleic acid is produced, collected on a filter, washed with alcohol and dissolved in just enough 10 per cent caustic soda solution. This is re-precipitated by pouring it into acid alcohol, and finally collected upon a filter.

346. A portion of the precipitated nucleic acid is taken in a test-tube with a few cubic centimetres of concentrated hydrochloric acid and a pinch of resorcinol; on heating, it gives an intense red coloration due to the presence of a carbohydrate group (pentose).

347. Phosphoric acid can be tested for in another portion which is hydrolysed in a test-tube by boiling for a few minutes with a few cubic centimetres of nitric acid diluted with an equal volume of water. Subsequently test with ammonium molybdate according to § 13.

### Hydrolysis of Nucleic Acid and Separation of Products of Hydrolysis

348. The nucleic acids can easily be hydrolysed by concentrated acids, with the employment of a moderate degree

of heat ; the solution so obtained is only slightly coloured and the decomposition products can easily be extracted. Take 5 grammes of commercial nucleic acid,<sup>1</sup> add 10 cc. of nitric acid of specific gravity 1.2 (6 cc. of acid (36° B.) and 4 cc. of water), so as to make a homogeneous mixture. Keep at a temperature of 25°-30°, shaking from time to time ; there is evolution of gas and the mass becomes liquid and transparent. The hydrolysis is complete in twenty-four hours.

349. *Separation of Purine Bases.*—For the separation of these, strong ammonia is added to the liquid (§ 348), diluted with 10 cc. of water, at first just to neutralization and then in considerable excess. A white precipitate of guanine results, which can be dried on the filter and purified by solution in 10 per cent hydrochloric acid and subsequent precipitation by ammonia.

350. The filtered liquid (§ 349) containing the bases other than guanine is freed from ammonia as far as possible by bubbling air through it, and then exactly neutralized with 10 per cent nitric acid. A saturated solution of sodium picrate is next added, this will give a precipitate of adenine picrate. This precipitate is collected and washed with water on a filter, and purified by crystallization from boiling water.

351. The remaining liquid, acidified with nitric acid, is extracted with ether, so long as the ether remains yellow, thereby removing excess of picric acid. A slight excess of ammonia is then added, and a 10 per cent solution of silver nitrate so long as a precipitate is produced. This precipitate containing the silver compounds of xanthine and hypoxanthine is drained on a filter, washed with a little dilute ammonia and again completely drained ; it is next transferred to a small flask together with 20 cc. of nitric acid and 20 cc. of water. A little urea is also added (to decompose nitrous acid). It is heated, and when ebullition starts, the heat is withdrawn and it is allowed to cool. The solution is filtered on a small asbestos plug ; the filtrate contains a silver compound of xanthine, while the precipitate contains hypoxanthine. The two bases can be separated from their

<sup>1</sup> This is obtained generally from beer-yeast.



silver combinations by decomposing these with dilute hydrochloric acid, filtering, and crystallizing out the hydrochlorides.

### General Reactions of Purine Compounds

352. The purine bases, adenine, guanine, hypoxanthine and xanthine, together with uric acid, are precipitated from their solutions as copper compounds by copper sulphate in the presence of sodium bisulphite. It is sufficient to add to 10 cc. of the liquid under examination, which has been rendered slightly alkaline by sodium carbonate, a few cubic centimetres of commercial solution of sodium bisulphite, then, shaking all the time, a solution of copper sulphate; the precipitation is aided by boiling. The copper compounds, which are white when perfectly pure, are usually slightly tinged with brown when they are formed in complex mixtures, and form gelatinous precipitates which readily clog the filters.

353. The purine bases and uric acid are precipitated by silver nitrate in solutions rendered distinctly alkaline by ammonia. These silver compounds form white precipitates insoluble in dilute ammonia, a reaction which is often used for their separation. Add to 10 cc. of the liquid to be tested enough ammonia to make it definitely alkaline, then, drop by drop, a 10 per cent solution of silver nitrate till no further precipitate occurs.

### Detection of Uric Acid and Caffeine

#### (*Murexide Reaction*)

354. This reaction is given by certain purine derivatives such as uric acid and caffeine. Into a small porcelain capsule (about 3 cm. in diameter) put a few milligrammes of the material to be examined, moisten with a drop of fuming nitric acid and evaporate off the excess of acid on a water-bath; finish the evaporation to dryness over a small flame. If the *thoroughly dry* residue is not coloured a brilliant rose

or orange, re-treat with nitric acid, cool and moisten with a small drop of dilute ammonia; the stain dissolves, giving a violet-purple colour. A drop of soda similarly dissolves it, giving in this case a blue solution.

355. The material can also be oxidized by a fragment of potassium chlorate and a drop of hydrochloric acid. Evaporate to dryness and treat with ammonia after cooling. In this last form the reaction is only given by uric acid, caffeine and xanthine.

### Reaction of Guanine and Xanthine

356. When a particle of guanine or xanthine is carefully heated with a drop of fuming nitric acid and evaporated, a lemon yellow stain is produced; this changes to orange on addition of a drop of soda solution. On now evaporating, it gradually reddens and may even show a violet tinge. The reaction is easily distinguishable from that given by uric acid under the same conditions. Adenine and hypoxanthine do not give colour reactions when treated in this manner.

### Estimation of Casein in Milk

357. Work with 10 grammes of milk, which have been treated (according to § 213) with a mixture of alcohol and ether to remove fat. The solution containing casein is precipitated by the addition of a 5 per cent solution of acetic acid, added drop by drop, with continuous shaking during the addition. (Such a volume of dilute acetic acid must be taken that it equals that of 1.5 per cent soda solution used subsequently to dissolve the casein.) When the precipitation is complete, centrifuge, decant the liquid and mix the precipitate with 20-30 cubic centimetres of water; centrifuge again and re-wash. The decanted liquids are passed through a weighed filter; finally 50-60 cc. of alcohol are added to the casein; this is then put on the filter, the whole washed again with alcohol and finally with ether. The filter and its contents are dried in the air-bath at

105° to constant weight. Finally the casein can be incinerated and the ashes weighed.

If boiled milk is used, coagulated albumin is weighed as well as casein.

### Estimation of Uric Acid and Purine Bases in the Urine

358. Human urine contains .3 to .8 gramme of purine bodies per litre ; three-fourths of this consists of uric acid, the remaining fourth of the bases xanthine, hypoxanthine, etc. When the proportion of uric acid is sufficiently high it is partly deposited on cooling as characteristic crystals

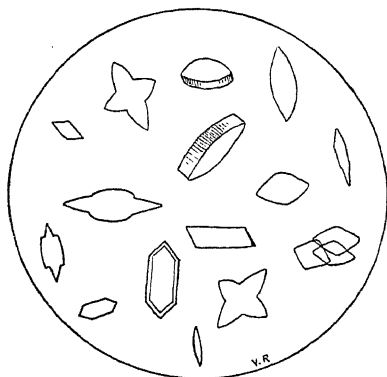


FIG. 33.—Uric Acid.

(Fig. 33). The uric acid and purine bases can be estimated together rapidly and sufficiently accurately for practical purposes as silver-magnesium compounds by means of a known volume of silver solutions added in excess ; the quantity of silver remaining in solution is then estimated (Haycraft's method, modified by Denigès).

359. A silver-magnesium solution is first prepared as follows : Dissolve 75 grammes of ammonium chloride and 50 grammes of magnesium chloride in 400 cc. of ammonia (22° B.), warming very slightly meanwhile. Add more ammonia to bring the volume to 510 cc., then filter into a

graduated litre flask containing 500 cc. of decinormal silver nitrate solution accurately measured (see § 63). When the flask is filled to the 1000 cc. mark and the contents thoroughly mixed, we have a N/20 silver solution.

360. Into a test-glass of 250 cc. capacity put exactly 25 cc. of the silver-magnesium mixture, then run in while stirring 100 cc. of urine; stir thoroughly again, then filter through a pleated filter-paper into a graduated 100 cc. flask. One thus has 100 cc. of liquid, corresponding to 80 cc. of urine; the silver content of this now has to be titrated. For this, put into a test-glass 10 cc. of N/10 solution of potassium cyanide,<sup>1</sup> and 1 cc. of a freshly prepared 10 per cent potassium iodide solution. Run in from a burette enough N/10 solution of silver nitrate (with continuous stirring) to form a slight permanent precipitate. The 10 cc. of potassium cyanide correspond exactly to the quantity of silver introduced at the beginning of the operation, hence the quantity of silver nitrate subsequently added corresponds to the amount used to precipitate the purine bodies. As each cubic centimetre of decinormal silver nitrate corresponds to 0.0168 gramme of uric acid, the total purine content (expressed in terms of uric acid), reckoned as grammes per litre, is given by the formula  $n \times 0.0168 \times \frac{1000}{80}$ , which works out approximately at  $n \times 0.21$ , where  $n$  represents the number of cubic centimetres of N/10 silver nitrate used.

361. To estimate uric acid by itself, in urine, the following technique may be adopted. The uric acid is precipitated as ammonium urate; this is dissolved in a medium rendered alkaline by sodium borate and potassium

<sup>1</sup> To prepare the decinormal solution of KCN, from 16.18 grammes of the pure commercial salt are dissolved in 1100 cc. of water, 10 cc. of soda solution (36° B.) are added, the whole well mixed and filtered if necessary. To standardize the solution, 10 cc. are mixed with an equal volume of ammonia (22° B.), and placed in a beaker with 50 cc. of water and 1 cc. of a 10 per cent solution of KI. The mixture is stirred, and N/10 AgNO<sub>3</sub> is run in until a faint permanent turbidity is produced. If  $n$  is the volume of AgNO<sub>3</sub> solution run in, then in order to make the cyanide solution exactly decinormal (*i.e.* exactly corresponding to the standard silver solution), a volume of water equal to  $(n - 10) \times 100$  must be added to 1 litre of the original cyanide solution. The solution keeps well, but its titre should be verified about once a fortnight.

bicarbonate, and is titrated by a decinormal solution of iodine (Ronchèse). To 100 cc. of urine add 15 cc. of ammonia (22° B.) and 15 grammes of ammonium chloride; leave for half an hour, then filter, washing the residue with a solution containing 150 cc. of ammonia and 150 grammes of ammonium chloride per litre. The filter is well drained, carefully unfolded, and the contents washed into a test-glass with a jet from a wash-bottle. The suspension is then introduced into a flask of 500 cc. capacity; add, drop by drop, 10 per cent acetic acid until the precipitate just dissolves; sufficient water to bring the volume to 300 cc. is next added; render alkaline by 20 cc. of a saturated solution of sodium borate which is also saturated with potassium bicarbonate, then run in a decinormal solution of iodine<sup>1</sup> till the liquid acquires a yellow tint which persists for a minute; the turning-point is, however, not very decided. Each cubic centimetre of N/10 iodine corresponds to 0.0084 gramme of uric acid, but as after the precipitation of ammonium urate, an amount of this salt remains in solution corresponding to 0.001 gramme of uric acid, the total uric acid content of the urine will be given by the formula  $10[(n \times 0.0084) + 0.001]$ : where  $n$  is the number of cubic centimetres of iodine solution employed.

### Estimation of Caffeine

362. Caffeine is a purine derivative (trimethylpurin), which occurs in variable proportions (1-3 per cent) in tea, coffee and maté. For its estimation boil 10 grammes of the substance under examination, finely powdered, in 100 cc. of water. After ten minutes, decant the liquid into a 500 cc. graduated flask, and repeat the extraction four times. To the liquid so obtained add lead subacetate in very slight excess to precipitate various matters foreign to the experiment in hand, make up to 500 cc., mix and filter. Take 400 cc. of the filtrate and add sulphuric acid, drop by drop,

<sup>1</sup> The N/10 iodine solution is made by grinding in a mortar 12.7 grammes of pure dry iodine with 26-30 grammes of KI and 10 cc. of water, and then bringing the total volume to one litre.

to precipitate the lead, filter, wash the precipitate three or four times, collect the washings and add them to the filtrate. Concentrate the mixed liquids, preferably by evaporation *in vacuo*, to 40-50 cc. ; filter the concentrated extract, wash out the flask and filter the resulting liquid ; mix the filtrates and extract with 40-50 cc. of chloroform. Repeat this extraction three or four times, using about the same quantity of fresh chloroform each time ; filter the mixed extracts. Distil off most of the chloroform from the mixed extract, transfer the residuum to an evaporating basin, and complete the evaporation on the water-bath. Caffeine crystallizes out ; dry it until it attains constant weight ; the final weight multiplied by 12.5 gives the number of grammes of caffeine in 100 grammes of material examined.

## CHAPTER XVI

### PIGMENTS

#### Hæmoglobin

363. HÆMOGLOBIN, the red colouring matter of the blood of vertebrates, is an iron-containing proteid, which is easily decomposed into a protein (globine) and a prosthetic group (hæmatin), which contains all the iron. For this decomposition it is sufficient to mix a few drops of blood with 5 cc. of water and to add a little hydrochloric acid; hæmatin then forms a brown flocculent precipitate. Hæmoglobin and especially its oxygen compound (oxyhæmoglobin) can, under certain conditions, be obtained in crystalline form; the form of crystal differs in blood from different kinds of animals.

#### Crystallization of Oxyhæmoglobin

364. To obtain crystals rapidly, the blood of the guinea-pig or rat is selected for preference. Guinea-pig blood gives crystals in the form of tetrahedra; rat blood gives very fine prismatic crystals, often grouped in rosettes. Horse blood gives very long crystals, and the blood of the squirrel regular hexagonal plates.

After collecting the blood in a small flask containing glass beads, shake vigorously for ten minutes, so as to facilitate the formation of fibrin which remains adherent to the beads, decant the defibrinated blood, and for each cubic centimetre add 7 or 8 drops of ether saturated with water, shake so as

to saturate the liquid and provoke hæmolysis (that is, the disintegration of the red-corpuscles which permits the solution of hæmoglobin), cork and stand on ice. Crystals are deposited in times varying from a few minutes to some days.

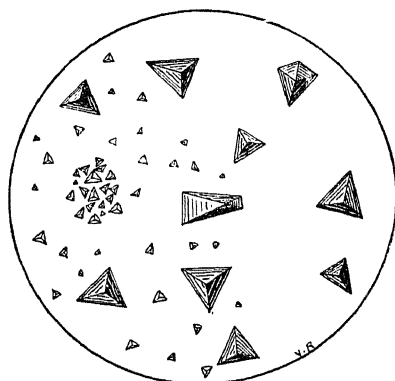


FIG. 34.—Oxyhæmoglobin Crystals (Guinea-Pig).

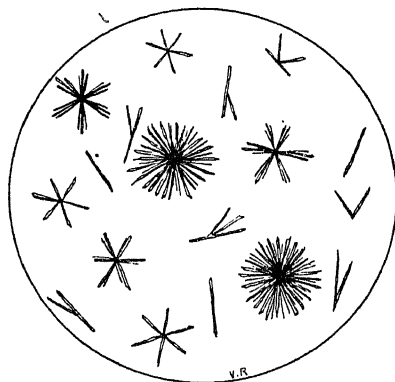


FIG. 35.—Oxyhæmoglobin Crystals (Rat).

365. If it is only desired to examine the crystalline form microscopically, mix on a glass slide a drop of defibrinated blood and a drop of water. Leave for a minute till the edges of the preparation begin to dry, then cover with a coverslip. Crystals will appear in the interior of the specimen about the inner margin of the dried ring.



### Preparation of Oxyhæmoglobin

366. When it is necessary to prepare crystallized oxyhæmoglobin in bulk, it is usual to employ horse-blood.

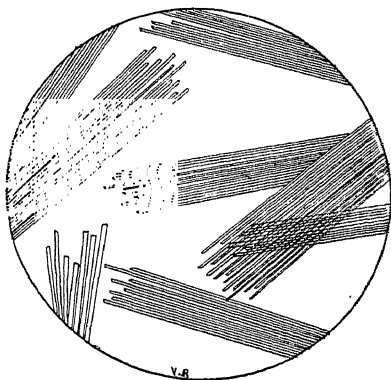


FIG. 36.—Oxyhæmoglobin Crystals (Horse).

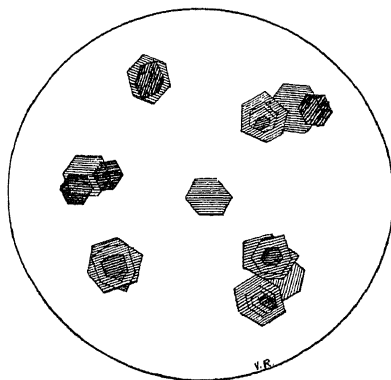


FIG. 37.—Oxyhæmoglobin Crystals (Squirrel).

Centrifuge the blood as soon as drawn, or after having rendered it non-coagulable by the addition of 1 gramme of powdered sodium oxalate per litre. After decanting the plasma, the corpuscles are taken up in .8 per cent solution of sodium chloride; centrifuge again, and repeat the centrifuging and washing twice more. The washed corpuscles are

mixed with two or three times their volume of warm water ( $40^{\circ}$ ) ; hæmolysis commences immediately, and the stroma of the corpuscles is removed by centrifuging. The red liquid is placed in a glass vessel surrounded by a mixture of ice and salt. When cooling is complete, add a quarter of the volume of alcohol cooled to  $0^{\circ}$ , and leave the whole in the freezing-mixture. Crystallization commences almost directly. After twelve hours drain the crystals on the filter-pump, and wash with a little 25 per cent alcohol cooled to  $0^{\circ}$ , then dry *in vacuo* over sulphuric acid.

### Examination for Blood by the Formation of Crystals of Hæmatin Hydrochloride

(*Teichmann's Crystals*)

367. Put on a glass slide (thoroughly cleaned) a small drop of defibrinated blood and a drop of 1 per cent sodium

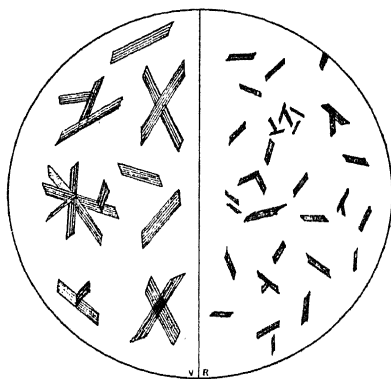


FIG. 38.—Teichmann's Crystals.

Right : Low power ; Left : High power.

chloride, mix and allow to dry at the ordinary temperature, or at any rate below  $40^{\circ}$ , so as to avoid coagulation of protein which would hinder the reaction. Put a large drop of glacial acetic acid upon the dried blood, put on a coverslip and warm gently over a very small flame, so as to just reach the boiling-

point of the acid. If the original red stain be carefully observed during the warming, it is seen to suddenly dissolve and to give place to a violet or bluish coloration. At this moment the transformation is accomplished, and the crystals can be observed under the microscope. Outside the zone where they first appear, the development of crystals in the rest of the preparation can be observed, especially near the edges of the coverslip, during the process of cooling.

### Spectroscopic Characters of Hæmoglobin

368. When a sufficiently dilute solution of oxyhæmoglobin (·2-·3 per cent), in a thickness of about 1 to 2 centimetres, is examined spectroscopically, a band is seen at the violet end of the spectrum, and also two absorption bands contained between the D and E lines; that nearer to the D line is the narrower, but it is also the more distinct of the two. When the direct vision spectroscope is adjusted as described in § 239, the D line corresponds with the wavelength 590, and the E line with 530.

369. Put into a test-tube 10 cc. of water and a few drops of defibrinated blood, shake, and when the mixture is transparent examine with the spectroscope. The two characteristic bands of oxyhæmoglobin are seen, whose centres correspond to w.l. 578 and 542 respectively.

370. Add to such a solution of oxyhæmoglobin, five to ten drops of some energetic reducing agent, *e.g.* sodium hydrosulphite,<sup>1</sup> a reduction takes place and reduced hæmoglobin is formed: the rose-coloured liquid becomes a deeper

<sup>1</sup> To prepare the hydrosulphite solution proceed as follows. Dilute 40 cc. of sodium bisulphite (36° B.) with 200 cc. of water, and add 5·6 grammes of powdered zinc. Cool the mixture, shake it well and leave for  $\frac{1}{2}$  hour. Then add milk of lime (prepared by mixing 6 grammes of quicklime with 30 cc. of water), and after two minutes filter. To the filtrate add 15 cc. of a 10 per cent solution of sodium carbonate. The liquid is then filtered once more, and transferred to small bottles, *which must be completely filled* and tightly corked. It retains its reducing powers for several days, especially if kept at a low temperature.

If this reagent is not available the reduction may be effected by means of ammonium sulphide solution, in which case reduction is slow and only complete after several minutes.

violet-red, and if examined spectroscopically, the two bands of oxyhæmoglobin are seen to approach one another and to be replaced by a single band, occupying approximately the region between the two oxyhæmoglobin bands. This is known as "Stokes' band," and its centre corresponds to w.l. 555. To observe the approach of the two bands and the formation of Stokes' band, the solution of oxyhæmoglobin should not be too dilute (10 drops blood to 10 cc.

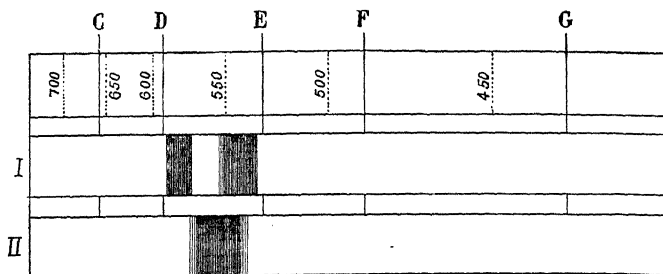


FIG. 39.

water at least), as the Stokes' band is always paler than the oxyhæmoglobin bands.

371. If instead of the reducing agent mentioned in the last paragraph, an oxidizer, such as potassium ferricyanide, is used (a few drops of a 10 per cent solution), methæmoglobin is formed and the solution becomes brown, and a third narrow band is seen to the red side of the D line.

### Reactions of Bile Pigments

372. The bile pigments, when treated with nitric acid containing a little nitrous acid, give a characteristic play of colours which succeed one another in a definite order—"Gmelin's reaction." To produce it, put about 5 cc. of concentrated yellow nitric acid into a test-tube, run on to the top of it by means of a pipette the solution containing bile pigments, so as to make two separate layers. In a few seconds a yellow and red ring appears at the junction of the

fluids, later come a series of superposed rings coloured from below upwards red, violet, blue and green.

373. Hammarsten has modified this reaction in the following manner. Prepare a mixture of 1 cc. concentrated nitric acid, 19 cc. of hydrochloric acid and 60 cc. of water ; leave this exposed to the light for a week. The liquid becomes a clear yellow and is then ready for use. Just before use a little is mixed with 95 per cent alcohol. To 2 or 3 cc. in a test-tube is added some drops of the solution of bile pigments ; a fine green colour is produced, which persists indefinitely. By adding to this green mixture increasing quantities of the alcohol acid mixture the green changes to blue, then to violet, red, and finally to yellow. Each of these tints thus produced keeps its colour indefinitely, unless more of the reagent is added. The whole series of colour changes can thus be prepared and kept.

### Bile Pigments in Urine

374. Put 10 cc. of urine into a test-tube, add 5 cc. of a 10 per cent barium chloride solution and enough barium hydrate solution to render the mixture alkaline, then shake well. Filter, and wash the precipitate with a little distilled water. Pierce the filter-paper and allow its contents to fall into a test-tube containing 5 cc. of alcohol (90 per cent) and 5 drops of hydrochloric acid (22° B.). Put the tube on a boiling water-bath for a minute and examine. If the urine contains bile pigments, the supernatant liquid is coloured a more or less bright green, according to the amount of pigment present. Sometimes a non-characteristic brown tint makes its appearance ; should this be the case, add two drops of hydrogen-peroxide and again warm on the water-bath ; then if bile pigments are present, the green colour appears. The reaction can be made still more marked, if the green alcoholic solution is decanted off, and is carefully run on to the surface (avoid mixing) of 5 cc. of nitric acid in a test-tube ; the characteristic play of colours, red, violet, blue and green is then seen (§ 372).

### Detection of Urobilin in the Urine

375. Urobilin is a pigment derived from the colouring matters of the bile, and which occurs in varying quantity in the urine. It is characterized by its power of giving a green fluorescence with alcoholic solutions of zinc salts.

376. Take about 1 gramme of zinc acetate in a test-tube and add 10 cc. of 96 per cent alcohol; add 10 cc. of urine, shake several times, then filter and allow the liquid to stand. Examine for fluorescence by illuminating the tube from as strong a source of light as possible, then examine spectroscopically. In the presence of urobilin notice the fluorescence and the absorption band in the spectrum between the E and F lines (between w.l. 480 and 530).

377. The fact that urobilin is not precipitated by mercury salts may also be used in its detection. Take 100 cc. of urine and treat it with 10 cc. of mercuric sulphate as in § 120. The filtered liquid is shaken with 10 cc. of chloroform in a separating funnel. The chloroform extract is filtered upon a small dry filter; collect it in a test-tube and add double its volume of a 1 per cent solution of zinc acetate in 96 per cent alcohol, but without mixing the liquids. At the line of separation a green ring appears if urobilin is present; on shaking, a green fluorescence makes its appearance.

### Pigments derived from Indole and Skatole in the Urine

378. Urine contains a derivative of indole, which on oxidation is converted into a blue pigment, soluble in amyl alcohol and chloroform. A derivative of skatole also occurs, which gives a red pigment on oxidation; this is insoluble in chloroform but soluble in amyl alcohol. To isolate these pigments proceed as follows. To 20 cc. of urine add 2 cc. of basic lead acetate solution, filter and take 10 cc. of the filtrate in a test-tube. Add 10 cc. of pure hydrochloric acid, then 2 cc. of chloroform, shake and allow to settle. If the chloroform is not coloured add two drops of hydrogen peroxide ("10 vols."  $\text{H}_2\text{O}_2$  diluted to 1/10 strength), shake

again and allow to separate; the chloroform will then be blue, more or less deep in tint according to the amount of indoxyl in the urine.

379. Remove the chloroform by a pipette, add a fresh 2 cc. of chloroform, and again extract; finally pipette off the chloroform extract, which should only have a faint blue colour. The remaining liquid is well shaken with 2 cc. of amyl alcohol; on separating, the amyl alcohol will have a red colour due to the skatole derivative.

### Preparation of a Solution of Chlorophyll

380. A solution of chlorophyll can easily be obtained by treating any kind of green leaves with strong alcohol; the solution is more rapidly made by warming on a water-bath. To obtain a very concentrated solution of chlorophyll, heat green leaves (nettle, spinach, etc.) with alcohol under a reflux condenser; when these are exhausted, repeat the operation with fresh leaves, and continue the operation until the extract is as strong as desired; the exhausted leaves should be removed before the fresh ones are added. It is better to work with leaves which have been allowed to dry, in order to avoid undue dilution of the alcohol, which would thereby lose much of its solvent power. The concentrated solution of chlorophyll is opaque, if examined in a thickness of 10-15 centimetres: in a less thickness, it appears red by transmitted light, and in a still thinner layer it shows a fine green colour.

### Spectroscopic Examination of Chlorophyll

381. A sufficiently concentrated chlorophyll solution shows more or less intense absorption bands, and indeed only allows light to pass in the middle of the green and in the extreme red. On diluting with strong alcohol, these bands gradually disappear, with the exception of a very distinct and well-marked band in the red about w.l. 660. This is still discernible even in high dilutions (1 : 10,000).

By acting on the chlorophyll solution with potash, a

marked red fluorescence is produced, while spectroscopically the absorption band is doubled, showing two black bands separated by a narrow interval of red. This is a very characteristic reaction of chlorophyll, and may be used for its detection or identification. We owe this observation originally to M. J. Chautard, who described it in 1874, but without specifying definitely the conditions under which it

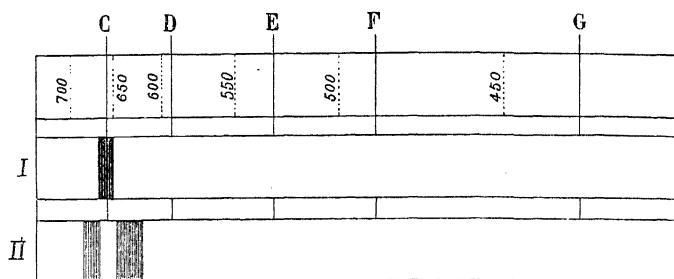


FIG. 40.—I. Absorption Band of Chlorophyll. II. Doubling of the Band consequent upon the Addition of Potash.

is produced. These conditions we have determined, and by carefully following the subjoined directions the phenomenon can easily be reproduced.

382. A convenient concentration of chlorophyll in small quantity is made by heating .25 grammes of fresh grass with 10 cc. of 96 per cent alcohol, in a test-tube filter with a cork and condensing tube, upon a water-bath for 20-30 minutes. The green solution, when put in a test-tube and spectroscopically examined, gives an intense black band in the red.

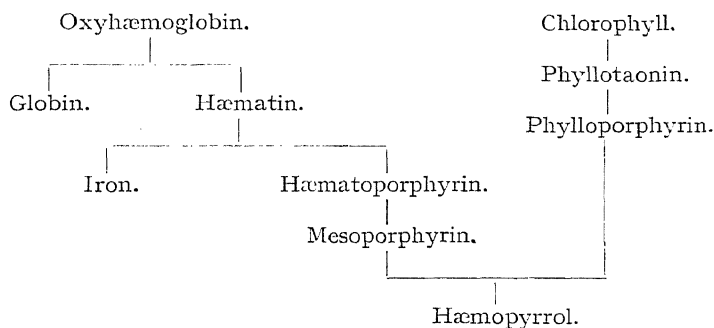
383. Then add 1 in 1000 of potassium hydrate (or about .1 cc. of 10 per cent alcoholic solution), bring to boiling and keep about that temperature for half a minute. On examining with the spectroscope, the original black band gradually doubles, and two black bands are seen separated by a thin red interval. When this point has been reached the reaction can be stopped by cooling the tube under a current of water. If, on the other hand, the reaction is allowed to continue, the band at the extreme red end of the spectrum becomes less distinct, while the other becomes more marked ; after a few



minutes the first band disappears and the second only is left, so that at this stage the action of potash seems to have displaced the original chlorophyll band towards the yellow. If the tube be cooled just when the two bands are most distinct, they persist quite a long time ; on adding dilute acetic acid till the liquid is distinctly acid, the two bands approach one another, forming a single band, but less clear and distinct than the original band.

### Products of Decomposition and Relations of Hæmoglobin and Chlorophyll <sup>1</sup>

384. Upon decomposition hæmoglobin gives successively hæmatin and then hæmatoporphyrin ; the latter body is isomeric with bilirubin and gives the same reactions. On the other hand, hæmatoporphyrin presents an obvious analogy to phylloporphyrin, which results from the decomposition of chlorophyll. Upon reduction, both hæmatoporphyrin and phylloporphyrin give rise to the same product, hæmopyrrol, which seems further to establish the relation between the pigment of vertebrate blood and that of green plants. The relations may be tabulated as follows :



<sup>1</sup> For a detailed summary of the extremely complicated questions involved in the question of the chemistry of chlorophyll, consult A. W. Stewart's *Recent Advances in Organic Chemistry*. Longmans, London, 1918.—[TRANSLATOR.]

### Extraction of Carotin from Green Leaves

385. Carotin is a hydrocarbon of a red-orange colour which co-exists with chlorophyll in green leaves. It is also found in the carrot and in the fatty coating of certain pollens, etc. To extract it, take the leaves of sorrel or chestnut (*e.g.*) and dry them as quickly as possible in a desiccator over sulphuric acid, *in vacuo*; after twenty-four hours the desiccation should be complete, and the leaves can be removed and easily powdered. Put the powdered leaves into a flask with a wide mouth, cover with petroleum-ether, cork the flask and leave for three days in the dark. Petroleum-ether does not dissolve chlorophyll, but extracts carotin and at the same time fatty and waxy matters, the extract having a yellow colour. Decant the extract into a photographic developing dish and allow to evaporate at room temperature.

The dried yellow extract will serve for the study of the reactions of the pigment. When it is taken up with a *little* petroleum-ether, the fatty matters are dissolved, the carotin remaining as small crystals with a reddish-brown reflection.

### Colour Reaction of Carotin

386. If a few cubic centimetres of carbon disulphide are added to a little of the extract, the yellow residue is dissolved, forming a blood-red solution. This solution, examined in a test-tube by the spectroscope, shows an absorption band from the green to the violet, one limit corresponding to w.l. 517 and the other to 486.

387. To another portion add a few drops of concentrated sulphuric acid; the residue is dissolved, giving a fine blue colour.

388. This second reaction can be used for showing the presence of carotin directly in the carrot. If a thin slice of carrot be taken, preferably from the cortical region, and examined under the microscope, reddish-orange needle-shaped crystals will be seen in the cells. These are crystals of phytostearin coloured by carotin, and they may also be

seen in a drop of liquid obtained by scraping a carrot. If a drop of concentrated sulphuric acid be put upon the preparation and examined at once after putting on a cover-glass, the crystals will be seen to have acquired a blue colour from contact with the acid.

389. When sufficient carotin is available, its di-iodide can be observed. This is obtained as a strongly coloured green flocculent precipitate on the addition of a freshly prepared solution of iodine in petroleum-ether to a solution of carotin (at least .01 per cent) in the same solvent.



# SECOND PART

## DYNAMICS

### CHAPTER XVII

#### HYDROLASES

#### **General Remarks on Enzymes**

390. THE preparation of solutions containing enzymes frequently involves more or less prolonged maceration of animal or vegetable tissues in water, glycerol, etc. Such maceration is preferably carried out at a low temperature with frequent stirring, and the tissues should be broken up or minced as finely as possible. The mixture is filtered, the first portions being filtered again until the filtrate is quite clear. Chardin's paper allows of the quickest and most efficient filtration. The liquid so obtained should be used immediately, as its powers undergo deterioration from exposure to air, light, and various other conditions.

391. The degree of enzyme activity is profoundly modified by certain chemical factors, such as alkalinity or acidity, and by physical conditions such as temperature. Each separate experiment must be performed under the conditions most suited to it. The optimum temperature, either known or determined experimentally, will be maintained by placing the experimental tubes in a water-bath (not an air-oven) fitted with a suitable regulating apparatus. Similarly the experiment will be conducted under the most

suitable conditions of acidity or alkalinity, verified at the beginning of each experiment, either volumetrically by titration in the presence of a suitable indicator (methyl orange, phenolphthalein, etc.), or electrically by a measurement of the hydrogen ion concentration.<sup>1</sup>

392. Nearly all solutions of enzymes are completely inactivated by a short exposure to a temperature of 100°. When investigating the properties of enzymes, a control experiment should always be made in which the enzyme solution has been heated to 100° on a water-bath for five minutes. Only such differences as exist between the heated and unheated specimens can be attributed to enzyme action.

In studying the products of enzyme action the qualitative examination should be supplemented whenever possible by a quantitative determination of at least one of the substances formed.

393. In conducting investigations of more than one or two hours' duration it is always necessary to guard against bacterial action, especially when working at temperatures of from 30° to 50°. Thus, when it is impossible to work aseptically recourse must be had to antiseptics which, while inhibiting bacterial activity, do not interfere with enzyme action. Sodium fluoride in concentrations of from 1 per cent to 2 per cent is often useful; chloroform used in such quantities that the liquid under investigation is saturated with it is also satisfactory, provided that the tubes are effectively closed so as to prevent evaporation. In this connection it must be remembered that chloroform reduces alkali-copper solutions; so that if a reducing sugar is one of the products under investigation all chloroform must be driven off by a few minutes' boiling before using this reagent.

394. According to the transformations which they effect, enzymes may be classified in the following manner (G. Bertrand):

1. *Hydrolases*: giving rise to decomposition with fixation of water (these are by far the most numerous).

<sup>1</sup> See p. 335.

2. *Oxidases*: causing the atmospheric oxygen to unite with certain compounds.
3. *Clastases*. Producing disruption of the molecule without addition of any sort.

### Preparation of a Solution of Sucrase from Yeast

395. Take 20 grammes of commercial compressed yeast and mix it in a mortar with 10-15 grammes of washed sand, gradually adding 5 cc. of water. Grind the mixture thoroughly for four or five minutes. Add 40 cc. of water little by little, mix thoroughly, and allow to stand for half an hour with frequent stirring. Filter carefully so as to eliminate any cellular debris in the filtrate, or, better still, separate by means of the centrifuge. When the yeast employed contains a considerable amount of glycogen the filtered or centrifuged liquid will be opalescent.

### Influence of Reaction and Temperature upon the Action of Sucrase

396. A 20 per cent solution of saccharose is prepared, and 10 cc. are put into each of a series of six test-tubes. A .3 per cent solution of acetic acid is next prepared, and the following mixtures are made and added to the sugar:

1. *Control*.—1 cc. boiled sucrase solution + 4 cc. water.
2. *Neutral*.—1 cc. active sucrase solution + 4 cc. water.
3. *Optimum Acidity* } 1 cc. active sucrase + 1 cc. of 1.5 per cent acetic acid + 3 cc. water.
4. *Acid*.—1 cc. active sucrase + 4 cc. of 1.5 per cent acetic acid.
5. *Alkaline*.—1 cc. active sucrase + 4 cc. decinormal soda.
6. *Neutral*.—1 cc. active sucrase + 4 cc. water.

The total volume in each test-tube is thus 15 cc., and each

tube should be distinctively marked so as to avoid any possibility of confusion. The tube 6 is left at the ordinary room temperature; the remaining five tubes are kept at 56° in a water-bath furnished with a regulator. After an hour the reducing power of the contents of each tube is determined. This can be done with sufficient accuracy by counting the number of drops necessary to decolorize 2 cc. of standard alkali-copper solution (see § 111). It will then be seen that the number is infinity for Nos. 1 and 5, very large for 4, less for 2, and the minimum for 3. It is greater for 6 than for 2.

Consequently it is seen that sucrase is more active at 56° than at the room temperature, and that its action is assisted by the optimum acidity of tube 3 (1 per 1000). It is inhibited by the stronger concentration of acid in tube 4, completely stopped by alkali (tube 5), and by previous boiling (tube 1).

### Influence of the Quantity of Sucrase upon the Hydrolysis of Sugar

397. The action of sucrase is proportional to the amount of enzyme employed, at any rate in the earliest stages of the experiment. To demonstrate this, four test-tubes are taken and 10 cc. of a 20 per cent solution of saccharose are put into each of them; then quantities of sucrase solution proportional to the numbers 1, 2, 3, and 4—*e.g.* .5 cc., 1 cc., 1.5 cc., 2 cc. are added to each tube, the volume of each of the first three tubes being made up to 12 cc. with water. The tubes are placed in the water-bath at 56°, and at the end of half an hour action is stopped by the addition of a few drops of soda solution. The amount of invert sugar in each tube is then determined.<sup>1</sup> The figures obtained will be proportional to the numbers 1, 2, 3, and 4—that is to say, to the amounts of enzyme used.

<sup>1</sup> Either by estimating the reducing power or by means of the polarimeter. In the latter case, instead of using soda to stop the reaction, 1 cc. of a 1 per cent solution of mercuric chloride may be used.



### Preparation of Emulsin from Almonds

398. From ten to a dozen sweet almonds are put into 50 cc. of boiling water for a minute. By this procedure the brown skins will be so loosened that they can be removed by pressure between the thumb and index finger. They are then pounded in a mortar, and when they are reduced to a paste, 30 cc. of water are added and then 1 cc. of 10 per cent acetic acid. This causes a coagulum of protein matter to form. The mixture is then filtered; the filtrate should remain clear when a drop of 10 per cent acetic acid is added to it. Should a precipitate form, more acid must be added, drop by drop, with constant stirring until no further precipitation occurs, and the filtration repeated. A perfectly clear and very active extract is produced in this way.

### Action of Emulsin on various Glucosides

399. In each of a series of test-tubes are placed 10 cc. of solutions of different glucosides, so that upon hydrolysis the same volume produces one molecule of glucose—*e.g.* :

Tube 1	10 cc. of 2·3 per cent amygdalin.
Tube 2	10 cc. „ 2·8 „ salicin.
Tube 3	10 cc. „ 2·7 „ arbutin.

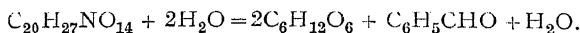
One cubic centimetre of emulsin solution is added to each tube. The tubes are kept in the water-bath at 45° for half an hour, and the reducing power of the contents of each tube estimated by counting the numbers of drops required to decolorize 2 cc. of standard alkali-copper solution.

It will be found that the rate of hydrolysis of different glucosides by emulsin is not the same; amygdalin is hydrolysed most rapidly, then salicin, and last arbutin.

It is necessary to ascertain that the solution of emulsin does not itself possess reducing properties, and in experiments where any degree of accuracy is required reduction due to glucose contained in the emulsin solution itself must be allowed for.

### Formation of Oil of Bitter Almonds

400. Bitter almonds contain both amygdalin and emulsin, which occur in different cells. When these are broken up the glucoside and enzyme come in contact, with the result that the glucoside is decomposed into glucose, benzaldehyde, and hydrocyanic acid (oil of bitter almonds), according to the equation—



To demonstrate this it is only necessary to skin some bitter almonds and pound them thoroughly in a mortar. The smell of benzaldehyde and hydrocyanic acid will be obvious almost immediately. Hydrocyanic acid can be identified by the following reactions :

### Properties of Hydrocyanic Acid

401. Hydrocyanic acid has the power of blueing test-papers treated with copper sulphate and tincture of guaiacum, and the action is very sensitive.

Filter-papers are soaked in a 1 in 1000 solution of copper sulphate and allowed to dry. Immediately before use the paper is moistened with freshly prepared guaiacum tincture ; on coming in contact, even with traces of hydrocyanic acid, an intense blue coloration is produced. This reaction, though exceedingly sensitive, is however also given by other bodies, notably by ozone, halogens, nitrous fumes, and ammonia.

402. *Detection of Hydrocyanic Acid.*—To make certain of the presence of hydrocyanic acid, the suspected liquid is distilled in a little flask with a crystal of tartaric acid. The use of mineral acids is inadmissible. The distillate is rendered alkaline by a few drops of 10 per cent soda solution, and tested for hydrocyanic acid as shown in §§ 7 and 8.

### Estimation of Hydrocyanic Acid

403. To estimate the amount of hydrocyanic acid contained in a given mixture, it is first of all distilled in a

current of steam and the resulting distillate titrated with silver nitrate.

If, for example, it is required to estimate the hydrocyanic acid liberated by crushing bitter almonds (§ 400), about 15 grammes of skinned almonds are ground up into a paste and put in a flask of 250 cc. capacity; the mortar is washed out twice with 25 cc. of water, and the washings are added to the contents of the flask, which is then well corked and allowed to stand for twenty-four hours. The reaction, which at first proceeds rapidly, is not complete until this time has elapsed. The flask is then fitted with a two-holed cork. Through one hole passes a bent tube, which is connected to the condenser; through the other passes a long tube reaching to the bottom of the flask, and conveying steam from a larger flask of 1 or 2 litres capacity. It is necessary to heat the flask containing the almond mixture gently, in order to avoid too great condensation of steam. At first the liquid froths a good deal, and consequently the connection between the steam generator and the distilling flask should be made with a rubber tube about 20 cm. long and not too narrow in bore. This allows of instant and easy disconnection whenever the froth shows indications of passing over into the condenser. As soon, however, as the protein bodies have been coagulated the distillation proceeds quietly.

The distillate is conducted by a bent delivery tube attached to the condenser to the bottom of a glass containing a mixture of 10 cc. ammonia, 10 cc. water, 1 cc. of a 10 per cent solution of potassium iodide, and 10-15 drops of strong soda solution.<sup>1</sup> During the distillation from start to finish a decinormal solution of silver nitrate is run into the above mixture from a burette until a faint turbidity is produced, the contents of the glass being of course constantly stirred. The cloudiness disappears as successive portions

<sup>1</sup> Since the distillate contains benzaldehyde, soda must be added to stop the turbidity which would ensue from the formation of benzamide and mask the cloudiness due to silver iodide. Soda need not be added if a simple aqueous solution of HCN is being distilled.

of distillate come over, and successive small quantities of silver nitrate are run in. When a faint opalescence persists for some seconds the current of water circulating in the condenser is shut off, in order that the steam ceasing to be condensed may drive over the last traces of HCN which remain in the atmosphere of the condenser. The operation is stopped as soon as steam tends to reach the titration mixture, which should not become heated. The end-point being reached, the distillation is now discontinued, since hydrocyanic acid has ceased to distil over.

Hydrocyanic acid in the presence of ammonia and a silver salt gives a double cyanide of silver and ammonium which is soluble in the presence of potassium iodide. When all the hydrocyanic acid has thus combined, further addition of silver nitrate produces insoluble silver iodide, which renders the liquid turbid and whose formation indicates the termination of the operation as above.

At this point each molecule of silver nitrate added has produced a double salt  $\text{AgCN} + \text{NH}_4\text{CN}$ ; hence each molecule of  $\text{AgNO}_3$  added corresponds to two molecules of HCN. Each cubic centimetre of decinormal silver nitrate is equivalent then to  $\cdot 0054$  grammes of hydrocyanic acid, and it is only necessary to multiply, by this factor, the number of cubic centimetres of  $\text{N}/10\text{AgNO}_3$  run in to give the amount of hydrocyanic acid present (Denigès).

404. The amount of amygdalin hydrolysed by enzyme action can be calculated by estimating the hydrocyanic acid liberated, since the crystallized glucoside has the formula  $\text{C}_{20}\text{H}_{27}\text{NO}_{11} + 3\text{H}_2\text{O}$ , and when completely hydrolysed yields 5.28 per cent of hydrocyanic acid.

### Demonstration of HCN-yielding Glucosides in Leaves

405. Leaves containing glucosides capable of yielding HCN, and enzymes which can decompose them, evolve hydrocyanic acid when subjected to the action of anæsthetics (chloroform, ether).

To demonstrate this action, take two cherry-laurel leaves

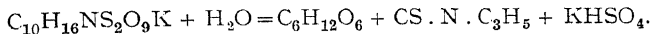
and place them in a couple of test-tubes which are filled two-thirds full with a mixture of equal parts of saturated picric acid solution and 1 per cent solution of sodium carbonate, so that the leaves are completely immersed. To one test-tube are added 10-15 drops of chloroform, the other remaining as a control. The tubes are next corked and placed in the water-bath at 35°-40°.

In a few minutes the liquid in the tube to which chloroform was added becomes reddened while the colour of the control tube remains unaltered. The reddening is due to the reduction of picric to picramic acid by hydrocyanic acid.

This reaction is of general application in testing leaves for the presence of glucosides yielding hydrocyanic acid.

### Distribution of Enzyme and Glucoside in certain Seeds

406. When the enzyme myrosin is allowed to act upon potassium myronate a decomposition occurs according to the following equation :



The allyl isothiocyanate or oil of mustard so liberated can be recognized by its odour.

Under certain circumstances the regional distribution of enzyme and glucoside can be demonstrated by this reaction. Thus the seeds of *Lunaria biennis* contain myrosin in the integuments and potassium myronate in the cotyledons (Guignard). To demonstrate this the seeds are made to swell by being wrapped in wet cotton-wool and left for twenty-four hours in the incubator at 30°. The integuments are then removed and put into a mortar while the cotyledons are transferred to another mortar. The contents of the mortars can be ground up separately, and no odour of mustard oil will be noticed. On mixing the contents of the mortars, however, the characteristic odour will be almost immediately perceived. A dozen seeds are sufficient for the experiment.

### Preparation of an Active Enzyme Extract from Malt

407. Malt, which is obtained by allowing grains of barley to germinate and immediately drying them, is very rich in enzymes. Commercial malt deprived of the little rootlets has very much the appearance of barley, but differs from it in that the grains are very friable and have a slightly aromatic odour.

An active extract which energetically converts starch into sugar can be made by grinding 20 grammes of malt in a small hand-mill and macerating it in five times its weight of cold water for four or five hours, stirring well from time to time. The mixture is filtered through a square of linen placed in a funnel, the residue being freed from liquid as far as possible by wringing the cloth. The liquid is now either filtered through a folded filter-paper, or, simply, centrifuged. The resulting extract contains amylase (a liquefying enzyme), and dextrinase (an enzyme producing sugar).

### Liquefaction of Starch Paste by Amylase

408. By exposure to a temperature of  $80^{\circ}$ – $85^{\circ}$  dextrinase undergoes rapid destruction while amylase is almost unaltered. If, therefore, the malt extract is allowed to act upon starch paste at this temperature the action observed will be almost entirely due to amylase.

The starch paste is prepared as follows : Six grammes of powdered starch are well mixed with 10 cc. of water and the mixture is added to 90 cc. of boiling water with thorough stirring. A homogeneous semi-solid paste results, which is allowed to cool to  $85^{\circ}$ ; at this temperature 1 cc. of malt extract is added and the mixture shaken. The paste liquefies rapidly and completely and is then boiled. Simple solution of the starch has occurred without conversion to sugar; it continues to give a blue colour with iodine and does not reduce alkali-copper solutions.

### Conversion of Starch to Sugar

409. If malt extract is allowed to act on starch at a temperature which does not exceed  $70^{\circ}$  two actions occur simultaneously: namely, liquefaction by amylase, and conversion to sugar by dextrinase. If the temperature is raised the yield of sugar is diminished, and in order to obtain as good a result as possible the temperature should be from  $65^{\circ}$  to  $70^{\circ}$ . The action is often stopped when the liquid no longer gives any coloration with iodine, but it should be noted that the formation of sugar proceeds after this point has been reached.

410. The stages in the conversion of starch to sugar may be observed in the following manner: one hundred cubic centimetres of 3 per cent starch solution (instead of 6 per cent as in the previous experiment) are prepared as before, allowed to cool to  $65^{\circ}$ - $70^{\circ}$ , and 2 cc. of malt extract (§ 407) are added, the whole being kept in a water-bath at  $65^{\circ}$ - $70^{\circ}$  during the experiment. A series of test-tubes are taken, and into each are put 10 cc. of water and 2 or 3 drops of a 1 per cent solution of iodine. Every two to four minutes (according to the activity of the malt extract) 1 cc. of the starch mixture is removed, added to one of the test-tubes, and the resulting colour noted. A series will then be obtained, ranging from an initial blue to colourless, the intermediate stages being violet, red, chestnut, and yellow. The presence of sugar (maltose) at the end of the experiment can be shown by the reduction of alkali-copper solution. By adding 4 to 5 volumes of alcohol to a sample of the liquid in the later stages of the action a white precipitate of dextrin will be seen.

411. *Production of Maltosazone.*—After precipitation of dextrin, as above, the liquid should be heated on the water-bath to drive off the alcohol. To obtain the characteristic crystals of maltosazone, take 10 cc. of the saccharified starch mixture and add 40 cc. of 96 per cent alcohol, filter, and evaporate off the alcohol on the water-bath. Transfer the residual liquid to a test-tube with 5 cc. of phenyl-

hydrazine acetate solution (§ 91), heat on the water-bath for an hour, and allow to cool. The yellow liquid will deposit crystals of maltosazone, which can be examined under the

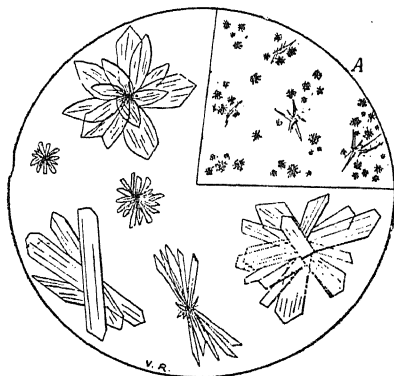


FIG. 41.—Maltosazone.

A. Low power.

microscope (Fig. 41). These crystals are freely soluble in boiling water and in methyl alcohol.

### Action of Salivary Amylase on Glycogen

412. Saliva contains an amylase which acts energetically upon starch and glycogen with the resulting production of maltose, dextrins being formed as intermediate products.

One gramme of glycogen prepared as in § 133 is dissolved in 5 cc. of warm water in a test-tube, and 5 cc. of filtered saliva<sup>1</sup> are added. After mixing, the liquid is kept at 40° in a water-bath for one hour, when a sample tested with iodine should give no coloration. On the addition of two volumes of alcohol dextrin is precipitated; on filtering and evaporating off the alcohol it will be found that the liquid has strongly reducing properties, and on suitable treatment yields crystals of maltosazone (§ 411).

<sup>1</sup> An abundant supply of saliva can be obtained by putting a drop of ether or chloroform on the tip of the tongue, collecting the saliva in a glass and filtering.



### Approximate Estimation of the Products of Saccharification by Observation of the Specific Gravity

413. A 10 per cent solution of glucose, maltose, or dextrin has a specific gravity of 1.0393, while a 1 per cent solution of either of these substances has a specific gravity of 1.0039 (water being 1.0). In dealing with such solutions the excess over unity is almost proportional to the concentration of dissolved material. If the gravity is taken at 15° with an accurate instrument the concentration of dissolved products is obtained by dividing the excess of density over 1.0 by 3.93 and multiplying by 1000. The method is applicable to mixtures of sugars as well as to the products of the action of enzymes upon starch; it is rapid, and taken in conjunction with estimation of the reducing power may be used for the analysis of mixtures of maltose and dextrin.

### Monobutyrimase of Blood-Serum

414. The blood-serum of various animals (rabbit, guinea-pig, horse, etc.) contains an enzyme capable of saponifying the esters of organic acids, such as the acetate or butyrate of amyl or ethyl, and the mono-esters of glycerol, especially monobutyrim. Since it has no action upon neutral fats, which are decomposed by the various lipases, it has received the name of monobutyrimase.

415. To demonstrate the action of blood-serum on monobutyrim it is only necessary to titrate the amount of butyric acid liberated. It will be found that the liberation of acid is at first rapid, but becomes markedly slower as saponification progresses, since the free acid exerts an inhibitory effect upon the reaction.

Five test-tubes are taken, and to each are added 10 cc. of a saturated watery solution of monobutyrim. To the first is added 1 cc. of horse-serum previously boiled with 1 cc. of water; to each of the four other tubes 1 cc. of un-boiled serum is added. To each tube is added one drop of phenolphthalein solution, and the contents are neutralized

with .5 per cent solution of sodium carbonate until a very faint pink colour is obtained. The series of tubes is then placed in the water-bath at  $35^{\circ}$ - $37^{\circ}$ .

After fifteen minutes it will be found that the contents of the control tube, to which boiled serum was added, remain neutral, while the contents of the other tubes have become acid. The number of drops of sodium carbonate solution necessary to neutralize the mixture in the first experimental tube is then found and noted ; suppose it to be  $n$ .

After a further fifteen minutes, approximately,  $n$  drops of soda will have to be added to the same tube to render it again neutral. If the second experimental tube be similarly tested it will require a quantity of soda which is less than  $2n$ , which shows that the action is not proportional to the time but progressively diminishes. This can be confirmed by observing tube 3 after forty-five minutes, and tube 4 after an hour. This slowing of the reaction is due to increasing concentration of free acid, since if it is neutralized the reaction proceeds as fast as it did at first.

416. The action of serum-monobutyrylase upon monobutyryl can also be measured by observing the diminution of surface tension which occurs as saponification progresses (Rona and Michaelis).

For this experiment 20 cc. of saturated watery solution of monobutyryl are mixed with 1 cc. of fresh horse-serum and 3 cc. of water ; the liquid is then placed in a Duclaux' drop-counting apparatus (§ 498), the lower opening of which is then dried and the number of issuing drops counted. This figure is in the neighbourhood of 160 and varies with the temperature. Such readings are repeated after fifteen, thirty, and forty-five minutes. It will then be observed that the numbers obtained for each fifteen minutes decrease regularly. By plotting the times as abscissæ and the number of drops obtained as ordinates, a curve illustrative of the course of the phenomenon can be constructed.

A control experiment should be performed. In this 4 cc. of serum previously diluted to one-quarter are heated

to 80° for an hour and added to 20 cc. of the monobutyryn solution. It will then be seen that the number of drops does not vary with the time that the serum and monobutyryn have been in contact.

### Saponification of Ethyl Acetate and Butyrate by Monobutyrynase

417. The esters of the monobasic fatty acids are saponified by monobutyrynase. This saponification occurs more readily with increasing molecular weight of the acid ; thus ethyl butyrate is saponified at least ten times as fast as ethyl acetate.

To prove this, 1 cc. of ethyl butyrate is emulsified by thorough shaking with 100 cc. of water. This is divided among several test-tubes, each tube receiving 10 cc. A drop of phenolphthalein solution is added to each and the contents neutralized by .5 per cent solution of sodium carbonate until a faint pink colour is produced. Next 1 cc. of fresh horse-serum is added to each, the contents of one tube being boiled, so that it serves as a control.

A similar series of tubes is prepared in which the place of ethyl butyrate is taken by a solution containing .75 cc. of ethyl acetate to 100 cc. of water ; this gives a solution which is equimolecular with that of the ethyl butyrate employed in the first series.

Both sets of tubes are placed in a water-bath regulated for 55°-60° ; it will be noticed that the control tubes retain their pink colour while the others are decolorized owing to the liberation of acid.

After five minutes a tube of butyrate and one of acetate are removed, cooled, and neutralized with .5 per cent sodium carbonate solution, the number of drops requisite for neutralization being noted in each case. Another pair of tubes is removed after the lapse of a further five minutes, and so on until all the tubes have been examined. Curves can then be constructed showing the intensity of saponification of the two esters in corresponding terms ; it will then

be seen that the decomposition of ethyl butyrate is very much more rapid than that of the acetate.

### Lipase of Castor Oil Seeds

418. Fat-containing seeds contain a lipoclastic enzyme which is almost inactive in a neutral medium, but which shows great activity in the presence of mineral or organic acids such as sulphuric or acetic. Castor oil seeds serve admirably for the study of this enzyme.

Five grammes (about 25-30) of these seeds are carefully skinned and pounded to a paste in a mortar. To this paste are added 7 cc. of castor oil<sup>1</sup> and 4 cc. of water, the whole being thoroughly mixed. The mixture is next transferred to a test-glass, and any fragments which remain in the mortar are removed with a piece of thin card.

A second mixture of seeds and oil is next prepared, but in place of water 4 cc. of 2.5 per cent acetic acid or of decinormal sulphuric acid are added. This mixture is turned into a glass as before, and both are allowed to stand at the room temperature for an hour. At the end of the hour 25 cc. of 95 per cent alcohol are added to each glass, the mixtures are stirred, and the acidity titrated with normal soda, a few drops of phenolphthalein being added as indicator. It will at once be seen that the acidity of the first mixture is very slight, while that of the second is considerable, even after allowance has been made for the acetic or sulphuric acid previously added, and which corresponds to about 1.5 cc. of normal soda. Indeed the acidity will correspond to 40-50 per cent of the acid originally combined in the oil.

If the mixture is heated to boiling on the water-bath for ten minutes before adding the acid it will be seen that the acidity does not vary. It is plain then that in this case

<sup>1</sup> Castor oil is so viscous at ordinary temperatures that its measurement with a pipette is impossible. If, however, it is warmed to 40° the viscosity diminishes sufficiently to allow of such measurements.

To clean vessels which have contained castor oil, they should be washed with strong alcohol, in which castor oil is freely soluble.

we are dealing with an enzyme action which is favoured by a slight initial acidity.

### Study of a Vegetable Milk-Curdling Enzyme (Rennin)

419. A milk-curdling enzyme or rennin is very widely distributed in the vegetable kingdom, and can be demonstrated in the juice obtained by the expression of the leaves, flowers or fruits, of a great variety of plants.

A typical example may be found in the common artichoke. The artichoke is first divested of its leaves or bracts and then cut up in a meat-chopper; the turbid juice is expressed by means of a press and filtered. Five test-tubes are taken and 10 cc. of milk placed in each, to four of which are added .5 cc., 1 cc., 2 cc., and 3 cc. of the juice; to the fifth tube, which is to serve as control, is added 1 cc. of boiled juice. The tubes are put in a water-bath at 50° and examined from time to time. Coagulation starts in a few minutes in the tube with 3 cc. of artichoke juice, and if the times necessary for coagulation to occur are noted, it will be seen that they are inversely proportional to the quantities of enzyme. Coagulation does not occur even after several hours in the tube to which boiled juice was added.

### Influence of Calcium Salts on Coagulation of Milk by Rennin

420. Milk coagulation by rennin is due to the fact that the casein, modified by enzyme action, forms an insoluble compound with calcium salts. If calcium salts are previously removed by some suitable precipitant (oxalate or fluoride of one of the alkali metals) the enzyme action occurs, but not the coagulation. The following experiment illustrates this point:

Into each of five test-tubes are put 10 cc. of fresh milk (to which no antiseptics have been added, since some samples of milk are thereby rendered non-coagulable), and

to the first tube is added 1 cc. of rennin solution<sup>1</sup> which has been boiled ; the milk in this tube will remain unclotted throughout the experiment.

The second tube receives .1 cc. of active rennin ; while to the third and fourth tubes .5 cc. of a 2 per cent solution of sodium oxalate is added, and after mixing .1 cc. of active rennin. The fifth tube receives only .1 cc. of a 10 per cent solution of calcium chloride. All the tubes are now placed in a water-bath at 35° for five or ten minutes, after which time it will be seen that clotting has only occurred in the second tube. The third and fourth tubes in which all calcium salts have been precipitated show no signs of clotting ; the casein has, however, undergone modification by the rennin, since if, *e.g.*, tube 3 be boiled a flocculent precipitate results which does not occur with untreated milk. If to the fourth tube (containing oxalated milk which has been subjected to the action of rennin) there be added 1 drop of calcium chloride solution, an immediate clotting occurs, although the clot is less compact than in tube 2. Tube 5, which contains only milk and calcium chloride, shows that this reagent alone is unable to effect clotting.

### Determination of the Clotting Power of Commercial Rennet

421. Commercially speaking, the " clotting power " of a sample of rennet means the relation between the volume of milk clotted and the volume of rennet used, the operation taking place at 35°. The figure is obtained as follows : Ten test-tubes are taken, and into each are put 10 cc. of milk. The sample of rennet is then diluted to 1 in 100, and of this dilution are taken .1, .2, .3, . . . 1.0 cc., and these amounts are put into the several test-tubes, the volumes of whose contents are made up to 11 cc. in all cases. The mixtures are shaken and put in the water-bath at 35°. Observations are made from time to time, and the time

<sup>1</sup> Commercial rennet or a 1 per cent solution of rennet powder.

necessary for *complete* clotting is noted—that is, when the tube can then be inverted without detachment or breaking of the clot. If the contents of one of the tubes appear to clot completely in forty minutes, the volume of milk and of diluted rennet which it contains are noted, and the ratio of the volume of rennet used to the volume of milk clotted gives the required “clotting power” of the sample. Thus if it occurs in the tube containing .4 cc. of the diluted rennet, it is  $\frac{10}{.004} = 2500$ , which is the figure required.

If all the tubes show clotting in less than forty minutes, a fresh series of tubes is taken and a dilution of 1 in 200 used instead of 1 in 100; if these tubes again all show complete clotting in less than forty minutes further dilutions must be used.

While performing the experiment it will be noticed that the clotting time is almost exactly inversely proportional to the volume of rennet used.

### Coagulation of the Blood

422. Spontaneous clotting of blood is due to the action of fibrin-ferment upon fibrinogen. This enzyme is produced by the action of the calcium salts of the blood upon a substance (pro-fibrin-ferment) which is secreted by leucocytes when removed from their normal conditions of existence. If the calcium salts of the blood are removed by the addition of oxalate, secretion of pro-fibrin-ferment by the leucocytes still occurs, but there is no coagulation. Thus if oxalated plasma is freed from cellular elements by centrifugalization it coagulates upon the addition of a few drops of calcium chloride solution.

The addition of sodium fluoride instead of oxalate has a different action, since not only are calcium salts precipitated, but secretion of pro-fibrin-ferment by the leucocytes is also inhibited. In this case the centrifuged plasma does not coagulate on the addition of calcium salts.

423. This may be shown by the following experiment :

The oxalated or fluorized plasma is obtained by receiving the fresh blood of an animal (horse, for example) in a glass containing 5 cc. of a 2 per cent solution of sodium oxalate, or 10 cc. of a 3 per cent solution of sodium fluoride for every 100 cc. of blood collected. (The usual proportion is .1 per cent of oxalate and .3 per cent of fluoride in the mixture.) After mixing, the blood is centrifuged and the plasma pipetted or siphoned off.

If now to 10 cc. of oxalated plasma there are added 10 drops of a 10 per cent solution of calcium chloride coagulation soon takes place, but if fluoride has been used this clotting does not occur. If, on the other hand, 10 cc. of fluorized or oxalated plasma are taken, and 1 cc. of serum containing active fibrin-ferment is added, coagulation takes place in both cases.

### Test for Commercial Pepsin

424. No standard has been agreed upon for the digestive power of pepsin. According to the French Codex, samples of commercial pepsin must conform to the following tests before they can be used in pharmacy :

A solution is made of the pepsin under examination (.1 gramme) in a convenient amount of 1 per cent hydrochloric acid (say 60 cc.), and 2.5 grammes of fibrin, dried at a low temperature, are added. (It is better to make the fibrin swell first by soaking it in a part of the acid.) The mixture is placed in a water-bath at 50°, and shaken from time to time; the fibrin soon swells up and then begins to dissolve. The fluid gives the precipitation tests for albumoses and a biuret reaction which increases in intensity as time goes on.

After six hours it is allowed to cool, then filtered, and 10 cc. of the liquid are removed. This is cooled to 15° under the cold-water tap, and should give no precipitate on the addition of 10-20 drops of dilute nitric acid. The other reactions of albumoses have disappeared, and only the peptone tests are given.



It is easier to employ fresh fibrin, prepared by washing the crude material (obtained from the slaughter-house), until it is colourless ; or fibrin preserved in glycerol<sup>1</sup> may be used.

If such preserved fibrin is used it must be thoroughly washed in water and then dried between layers of filter-paper ; 10 grammes of this fibrin are used and the pepsin is dissolved in a mixture of water (46.5 cc.) and 10 per cent hydrochloric acid (6 cc.) ; this allows for the quantity of water still remaining in the fibrin.

It must be understood that the above is a purely arbitrary test for pharmaceutical purposes ; it takes no account of the nature of the products of digestion, and only furnishes a limit of activity below which samples are inadmissible for therapeutic purposes.

425. To compare the activities of different samples of pepsin, they must be allowed to act under standard conditions upon an excess of serum, and the liberated amino groups titrated by Sørensen's method (§ 319).

For this purpose 10 cc. of horse-serum are taken, to which .7 cc. of normal hydrochloric acid is added. The acid and serum must be accurately measured in pipettes so as to give a free acidity corresponding to 2.5 per 1000 of hydrochloric acid. A measured quantity of pepsin solution is then added ; this must, however, not be in sufficient amount to digest all the protein present (*i.e.* some albumoses precipitable by saturation with ammonium sulphate must remain at the close of the experiment). Digestion is allowed to go on at 37° for a certain number of hours, and then the mixtures are titrated by Sørensen's method. The activities of different preparations may be considered as proportional to the quantities of alkali necessary to neutralize the carboxyl groups.

<sup>1</sup> It is best to use 3 or 4 parts of glycerol to each part of fibrin in order to avoid auto-digestion of the fibrin, which will occur if it is insufficiently dehydrated. Even under these conditions preservation is only assured for a few months.

### Test for Commercial Trypsin

426. A mixture of 2.5 grammes of dry fibrin and 60 cc. of water, in which are dissolved .2 gramme of trypsin, is allowed to digest at 50° in the water-bath. It is shaken from time to time, and it will be noticed that the fibrin is gradually dissolved while the liquid gives the reactions of albumoses. At the end of six hours these reactions are no longer given, and on the careful addition of dilute nitric acid no turbidity is produced, peptone reactions only being given.<sup>1</sup> On continuing the digestion it will be found that samples give a gradually diminishing biuret reaction and, on cooling, deposit crystals of tyrosine which can easily be recognized under the microscope (§ 314).

Fresh fibrin washed and dried between filter-papers can also be used in this experiment; for this purpose 10 grammes of fresh fibrin and 52.5 cc. of water are employed; this allows for the water contained in the fibrin.

As in the case of peptic digestion, the process of tryptic digestion can be followed by titration of the resulting amino-acids by Sørensen's method (§ 319).

427. It is noteworthy that commercial trypsin contains amylolytic as well as proteolytic enzymes. Their activity can be estimated by mixing a solution of .01 gramme of "pancreatin" in 10 cc. of water with 90 cc. of 6 per cent starch paste cooled to 55° and kept at that temperature. The starch paste is soon liquefied, and the liquid rapidly acquires reducing powers. The production of maltose and dextrin can be verified as in § 410.

### Action of Papaïn on Serum Proteins

428. Papaïn has a digestive action which rapidly increases with rise of temperature, even up to 90°. It conse-

<sup>1</sup> This method prescribed by the Codex of 1908 does not give complete digestion of the fibrin; a portion remains undissolved and the albumoses do not disappear in the scheduled time. It is better to replace the water in the above experiment by very dilute soda (N/100) or sodium carbonate solution (1 gramme crystallized salt to 1 litre).

quently follows that if protein is heated in its presence to 100° a considerable amount of digestion takes place during the heating to boiling.

Two test-tubes are taken, and into each are put 2 cc. of a 5 per cent solution of papain ; one tube is heated to 100° for two minutes. To each tube are next added 10 cc. of *fresh* horse-serum, and both are put in a water-bath which is then heated to boiling. They are kept in this until a thermometer placed in the tube containing the unboiled papain indicates 98°-99°. Both tubes are now withdrawn : one will be found to contain a slightly turbid liquid, the other (which contains the previously boiled enzyme) is coagulated. If the contents of both tubes are now filtered, the filtrate from the latter (containing boiled papain) is very poor in protein substances—*i.e.* there has been no digestion.

The tube to which the active unboiled enzyme was added contains a fluid rich in albumoses, and no coagulum remains on the filter if the digestion has been rapid and complete.

Commercial papain rapidly deteriorates in strength, consequently only recent preparations should be used.

### Vegetable Urease

429. A very active urease can be extracted from many seeds, notably from those of the soya (*Glycine hispida*). The seeds are ground up in a mill, deprived of fat, and allowed to macerate in water.

Ten grammes of the seeds are taken and either pounded in a mortar or ground in a coffee-mill. The ground product is allowed to macerate for half an hour in petroleum ether. This is then carefully decanted and the operation repeated for a further half-hour. The extraction is repeated five or six times, the fatty matter being thereby removed. The residue is dried between filter-papers and put in the 35° air-oven for an hour or two. The dried powder is next macerated in five times its weight of water for 4 to 6 hours,

being shaken from time to time and then filtered, when a clear liquid is obtained which is very rich in urease.

430. To obtain an idea of the activity of the liquid so obtained 10 cc. of a N/5 solution of urea (or 1.2 per cent) are added to each of six test-tubes. To five of these are added 1 cc. each of the urease solution; the sixth tube receives 1 cc. of the same liquid which has been boiled. The tubes are then left at the ordinary temperature ( $20^{\circ}$ - $25^{\circ}$ ). After 5, 10, 15, 20, 25 minutes respectively the tubes are removed and 2 cc. of normal sulphuric acid added; they are boiled to expel carbon dioxide, and neutralized with decinormal soda solution in the presence of methyl orange. The amount of ammonia which has been formed from the urea by the action of urease can then be readily calculated.

## CHAPTER XVIII

### OXIDASES

#### Preparation of a Solution of Laccase

431. A SOLUTION of laccase can readily be prepared by macerating certain fungi (especially *Russula* and *Lactarius*) in glycerol. Failing these, ordinary mushrooms may be used, the lower half of the stalk being selected.

Fungi, containing large amounts of laccase, are easily recognized by allowing a drop of fresh guaiac tincture<sup>1</sup> to fall upon the freshly cut surface, when a vivid blue colour appears. To prepare a glycerol extract the fungi are first carefully skinned, broken into small fragments, and put in a wide-mouthed bottle with twice their weight of glycerol (S.G. 1263). The bottle is corked and placed in the dark for a few days.

When it is desired to use the liquid a little is filtered, but the bulk is kept in contact with the broken fungi, since under these conditions it retains its activity much longer than when filtered.

#### Colour Reactions of Laccase

432. Laccase acts upon a certain number of polyatomic

<sup>1</sup> Guaiac tincture is prepared by dissolving 5 grammes of guaiac resin in 60-70 cc. of 96 per cent alcohol with the aid of gentle heat. The mixture is filtered and about 30 cc. of water are added. It should be made fresh as required. If kept it undergoes oxidative changes, so that when mixed with water it forms an emulsion which turns blue on the addition of a peroxidase in the absence of hydrogen peroxide. This source of error must be carefully guarded against.

phenols in the presence of oxygen, and converts them into quinone derivatives. Phenols which readily yield quinone derivatives are the most susceptible to the action of laccase. Thus the *para* compounds (hydroquinone) are most easily oxidized; then come the *ortho* compounds (guaiacol, pyrogallol), while *meta* compounds are hardly affected at all.

Phenol compounds, in which the hydroxyls are wholly or partially replaced by  $\text{NH}_2$  groups, behave in the same manner.

433. Some compounds are oxidized very rapidly; among them are guaiaconic acid (contained in guaiac resin) and guaiacol; the former yielding a fine blue derivative, while the latter gives tetraguaiacoquinone, which has a garnet-red colour. To show this .5 cc. of laccase solution is put into each of four test-tubes with 2 cc. of water. Two tubes are boiled to serve as controls. Into one pair of tubes are put 10 cc. of water and 1 cc. of fresh guaiac tincture; on mixing, the tube with the boiled extract remains milky white, while the other gradually acquires an indigo-blue colour.

To the other pair of tubes are added 10 cc. of a 1 per cent solution of guaiacol. The control remains colourless; the other gradually reddens, owing to the formation of tetraguaiacoquinone which is finally precipitated as a crystalline powder.

434. Both hydroquinone and pyrogallol are oxidized by laccase to definite chemical compounds. Hydroquinone gives quinone which combines with excess of the phenol to produce quinhydrone, which, owing to its low solubility, is precipitated as scales with a reddish-brown reflection. Pyrogallol gives purpurogallin, a yellowish-brown powder which is also very slightly soluble.

These reactions may be obtained by mixing 10 cc. of a 2 per cent solution of hydroquinone or of a 1 per cent solution of pyrogallol with .5 cc. of the glycerol extract of *Russula* or other suitable fungi. A yellow colour soon appears, and after some hours a precipitate of quinhydrone or purpurogallin appears.

Purpurogallin gives a yellowish solution with water, which turns dark blue on the addition of ammonia.

### Action of Acids upon Laccase

435. Regarded from the standpoint of their action upon laccase, acids fall into two groups. The first, comprising such acids as sulphuric, hydrochloric, acetic, oxalic, etc., are strongly inhibitory, even when very dilute. The second, of which carbonic and boric acids are examples, are almost inactive even in high concentrations, and to these latter may be added certain acid salts such as mono-potassium phosphate, di-sodium citrate, etc. It may be noted that the acids which have a strong inhibitory action upon laccase also react with helianthin; while those acids and acid salts which are inactive towards laccase are also without action upon this indicator. Equimolecular solutions of the different active acids have identical actions.

436. Take a series of 5 test-tubes, and into each put 10 cc. of a 1 per cent solution of guaiacol. Three tubes then receive respectively 1, 2, and 3 drops of  $N/20$   $H_2SO_4$ ; the fourth 10 drops of a solution containing 10 per cent each of mono-potassium phosphate and di-potassium phosphate; to the fifth, which serves as control, no addition is made. Finally, to each of the five tubes of the series is added .1 cc. of a 2.5 per cent laccase solution, extracted from the latex of the lac-tree. The contents of each tube are well mixed, and in five or ten minutes a red colour appears in tubes 4 and 5 (to the first of which the phosphates were added, while the second was left as control without addition). Subsequently it makes its appearance in tube 1, while tubes 2 and 3 remain colourless. If now the contents of tubes 2 and 3 be tested with litmus-paper it will be found that they do not redden it, but by adding to each 10 drops of the mixed phosphate solution (itself acid to litmus) the action of laccase manifests itself. A red coloration soon makes its appearance, because the free acid has been replaced by acid phosphate which does not interfere with the reaction.

437. This inhibition of the action of laccase by acids is much less marked in the presence of certain salts. If the glycerol extract of (*e.g.*) *Russula* be used it will be found necessary to increase the amount of acid, when salts are present, in order to retard or inhibit the reaction.

The experiment described in the previous paragraph may be repeated with five tubes containing 10 cc. of 1 per cent guaiacol, but to each tube are added respectively 1, 2, 3 drops of *normal* (not N/20 as before) sulphuric acid. Four drops of the laccase solution, filtered immediately before use, are then added to each tube. The red colour will not be produced in the tubes containing free sulphuric acid until after the addition of 10 drops of the phosphate solution (§ 436).

### Preparation of Tyrosinase from Bran

438. Bran obtained from wheat contains tyrosinase but not laccase. The tyrosinase is easily extracted by macerating 1 part of bran with 4 parts of chloroform water in a well-stoppered bottle, which should be *filled* with the liquid. After four or five hours the contents of the bottle are strained through a cloth and the fluid remaining in the residue is pressed out. The resulting fluid is centrifuged, and the clear supernatant liquid is precipitated by the addition of three times its volume of 96 per cent alcohol. The precipitate is separated by the centrifuge, washed with a little 80 per cent alcohol, and taken up in a small quantity of water. Some of the protein materials coagulated by the alcohol are insoluble in water; these are again separated by the centrifuge. The supernatant liquid is once more precipitated by the addition of three or four times its volume of alcohol; the precipitate is then collected and dried *in vacuo* over sulphuric acid.

This precipitate is freely soluble in water; its solution is without action on an emulsion of guaiac resin or upon a solution of guaiacol, but in the presence of air it produces in tyrosin solutions a range of colours from rose, through red and brown, to black.



### Action of Tyrosinase upon Tyrosine

439. The action of tyrosinase can be studied by using either preparation made from wheat-bran (§ 438), or glycerol extract of certain fungi (notably *Russula*), which contains a large amount of tyrosinase.

Into four test-tubes are put 10 cc. of a cold, saturated, watery solution of tyrosine, and to these are added .1, .2, .3, .4 cc. of the glycerol extract. At the end of a certain time (to be noted) a rose colour appears in the tubes; these times will be found to be inversely proportional to the volumes of extract added. In a few moments the colour becomes red, and after several hours black. In the last case the bottom layers still remain red, showing that oxidation is most active in the superficial layers.

A control experiment, in which .5 cc. of extract is boiled with 2 cc. of water and then added to 10 cc. of tyrosine solution, shows no coloration.

Tyrosinase can also oxidize a certain number of monohydric phenols, such as ordinary phenol, paracresol, and several amino-phenols.

### Distinction between Peptones obtained by Tryptic and Peptic Digestion by means of Tyrosinase

440. Peptones obtained by tryptic can be distinguished from those obtained by peptic digestion by the colour produced when tyrosinase is added to their solutions (Harley). The peptic products give a red colour, which turns green after a short time; tryptic products, on the other hand, give a red, which in a few hours becomes brown, and finally black. The liquid should be completely neutralized before addition of the enzyme, otherwise these colour reactions are not given.

The tryptic peptones, moreover, give the reaction of tryptophane with bromine water.

### Separation of Laccase and Tyrosinase by Heat

441. Glycerol extracts of fungi contain both laccase and tyrosinase. The two enzymes can readily be differentiated by the action of heat ; tyrosinase being easily destroyed by heat, while laccase is very resistant. Specimens of tyrosinase differ in their susceptibility to heat according to their origin ; thus that obtained from the common mushroom is destroyed by three minutes' heating at  $65^{\circ}$ - $70^{\circ}$ , that from *Russula queletii* at  $70^{\circ}$ - $75^{\circ}$ , while tyrosinase from bran is only inactivated at from  $90^{\circ}$ - $95^{\circ}$ .

442. To effect a separation of the oxidases contained in a glycerol extract a series of test-tubes is taken and 1 cc. of the extract is added to each. Three of these tubes are placed in the water-bath at  $65^{\circ}$ , and when a thermometer immersed in the liquid in one of the tubes indicates this temperature, they are allowed to remain for exactly three minutes. The tubes are then removed, and they receive respectively 10 cc. of .1 per cent tyrosine solution, 10 cc. of 1 per cent guaiacol, and 10 cc. of a mixture of 2 cc. tincture of guaiac with 8 cc. of water. It will be seen that the first tube (containing tyrosine) remains colourless or is only faintly tinged, while the second becomes red, and the third blue.

If the tyrosinase is found not to be destroyed by heating, as above, the heating should be repeated with a fresh specimen at a temperature of  $70^{\circ}$ , and so on until the tyrosinase is found to be inactivated. The laccase present will, however, still be able to effect the oxidation of guaiac resin and guaiacol, thereby demonstrating its greater resistance to heat.

### Accurate Demonstration of Oxidases

443. Since the chief property of oxidases is the fixation of atmospheric oxygen by certain oxidizable substances in their presence, their accurate identification is based upon this fundamental character.

The following little apparatus is useful in this connection. It consists of a tube B, like a test-tube, but furnished with two constrictions E and E', to which is fused a narrower side-tube A terminating in a little bulb (Fig. 42). A few drops of the solution under examination are placed in A and 5 cc. of an oxidizable solution (*e.g.* guaiacol 1 per cent or tyrosine 1 per cent) in B. The little glass cover C is then

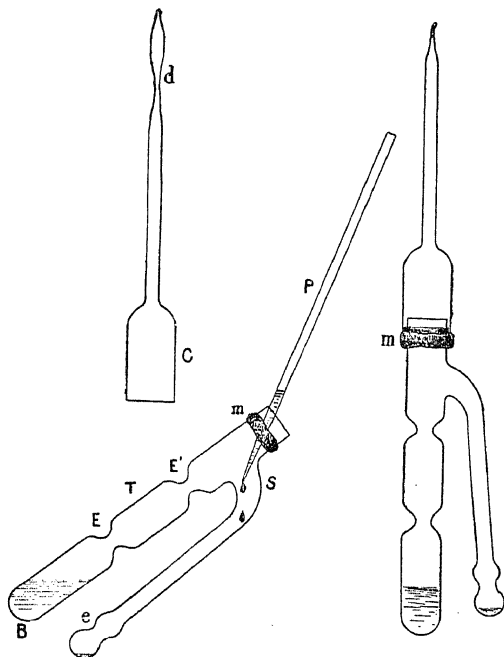


FIG. 42.—Apparatus for Investigation of Oxidases.

fixed over the apparatus by a ring of Golaz' wax; this cover carries a tube some 20 cm. long and drawn out at *d*. When the apparatus is adjusted it is connected up to a T-piece connected on the one hand to a filter-pump and on the other to an apparatus for delivering carbon dioxide. It is first exhausted by connecting to the pump, and then carbon dioxide is allowed to enter. The apparatus is slightly shaken to saturate the liquid, then the tap is closed and it

is again exhausted. This is repeated three or four times so as to evacuate any contained oxygen. (If a mercury pump is available one such procedure is sufficient.) Finally, it is exhausted as completely as possible and closed by the application of a small flame at *d*. On now manipulating the apparatus so that the contents of A and B are mixed, no coloration results, even though they be left for several days; if then the fine point be broken so as to admit air, coloration ensues in the presence of an oxidase (*e.g.* laccase acting upon guaiacol, or tyrosinase upon tyrosine).

## APPENDIX

### PEROXIDASE

#### Action of Hæmoglobin

444. The hæmoglobin of the blood behaves in the presence of a solution of hydrogen peroxide in the same manner as laccase in the presence of air, so that a mixture of hæmoglobin and hydrogen peroxide gives all the colour reactions of laccase.

In a liquid wherein the presence of blood is suspected this peroxidase reaction may be tested for as follows. To 10 cc. of the liquid is added 1 cc. of fresh guaiac, the emulsion remains white; but on addition of a drop<sup>1</sup> of neutral hydrogen peroxide solution a blue colour develops, which is one of the many possible reactions of blood.

#### Application of Peroxidase Reaction to Differentiation of Raw and Cooked Milk

445. Milk has a slight peroxidase reaction which it loses

<sup>1</sup> In investigating peroxidase and catalase it is essential to work with *neutral* hydrogen peroxide. This is prepared by adding to commercial hydrogen peroxide ("10-12 vol.")—which is always definitely acid—enough baryta water to render it faintly alkaline. A single drop of sulphuric acid is added, so as to render it *very faintly* acid; it is then filtered to remove the precipitate of barium sulphate.

When the acidity of a sample of hydrogen peroxide is due to boric acid, it is unnecessary to neutralize, since boric acid is without inhibitory action upon these enzymes.

upon boiling ; by this means uncooked milk can be distinguished from that which has been boiled.

Four test-tubes are taken, and into each are put 10 cc. of milk ; two are boiled for thirty seconds *and then cooled*. The cooling must be complete, since *warm* boiled milk gives the same colour reactions as raw milk.

One tube of boiled and one of fresh milk each receive .5 cc. of fresh guaiac tincture ; no visible reaction occurs. If to each a drop of hydrogen peroxide (4 or 5 vols. or over) is added, a blue colour develops in the raw specimen, the boiled remaining unaltered.

The reaction is rendered more distinct if a mixture of tincture of guaiac with a little guaiacol is employed. By adding to 10 cc. of raw milk .5 cc. of guaiac tincture and two drops of a 5 per cent alcoholic solution of guaiacol, a deep blue colour is immediately produced.

To the remaining two of the original four test-tubes are added 4-5 cc. of a 1 per cent solution of guaiacol ; both remain unchanged. On the addition of a drop of hydrogen peroxide the contents of the tube containing raw milk are reddened, the boiled specimen remaining unchanged.

#### Detection of Peroxidases in the Presence of Oxidases

446. For this purpose the little apparatus described in § 443 may be employed. In the tube A is placed the solution under examination, and in tube B a few cubic centimetres of guaiacol with one drop of pure hydrogen peroxide solution. The air is exhausted, as previously described, the apparatus being then manipulated so as to mix the liquids in A and B. In the presence of a peroxidase the red colour appears even in the absence of air ; the reaction indeed corresponds to an oxidation phenomenon which results, not from the activity of the atmospheric oxygen, but to atomic oxygen liberated from hydrogen peroxide.

## CHAPTER XIX

### CLASTASES

#### Preparation of Zymase from Yeast

447. AN extract which is rich in zymase can be prepared by macerating in water yeast which has been dried under certain special conditions (A. Lebedeff). The method is, however, not applicable to all strains of yeast.

448. *Desiccation of the Yeast.*—Fresh brewers' yeast is mixed with four to five parts of cold water. It is then allowed to stand, and some scraps of ice are added; this accelerates the settling of the yeast. When it has settled, the supernatant liquid is decanted off, the yeast drained on a cloth and then gently pressed, first by hand and then in a press. At the end of the procedure it is white and friable, and can be sifted through a large-meshed sieve. It is then set to dry thoroughly upon sheets of paper in an air-oven at  $35^{\circ}$ - $37^{\circ}$ ; this will require about two days. The resulting material is powdered and preserved in a dry bottle.

449. *Preparation of the Extract.*—A hundred grammes of this dried yeast are mixed with 300-350 cc. of water, and the mixture left at  $35^{\circ}$ - $37^{\circ}$  for three or four hours, with occasional shaking. It is then filtered through Chardin's filter-paper. The opalescent yellowish filtrate contains zymase.<sup>1</sup> The presence of zymase can be demonstrated by taking 1 gramme of finely powdered saccharose in a test-tube with 10 cc. of the above liquid. The tube is inverted

<sup>1</sup> It also contains large quantities of other enzymes, sucrase, catalase, etc.

two or three times without shaking (to avoid froth), in order to dissolve the sugar, and then allowed to stand. After some minutes small bubbles of gas appear which arise from the bottom and sides of the tube. The evolution of gas ( $\text{CO}_2$ ) gradually increases, and in half an hour a white froth over a centimetre deep is formed. The liquid remains clear, and it is evident that the fermentation is not due to the presence of cells which have been accidentally introduced.

After two or three hours the liquid is diluted with ten parts of water and distilled; the first portions of the distillate contain alcohol which can be detected by the reactions given in §§ 493 *et seq.*

### Preparation of a Solution of Animal Catalase

450. *Catalase of Adipose Tissue.*—From 10 to 15 grammes of raw pork fat are crushed up in a mortar with their own weight of water for two or three minutes, and the mixture is then filtered. The filtrate, when added to its own volume of hydrogen peroxide, produces a lively evolution of free oxygen. If the extract is previously boiled for a minute no oxygen is liberated when it is added to hydrogen peroxide.

451. *Catalase of the Liver.*—A hundred grammes of calf's liver are ground up in a mortar, with a sufficiency of white sand, and mixed with 50-60 cc. of 96 per cent alcohol. The mixture is allowed to stand for a quarter of an hour, and then expressed as forcibly as possible. The residue is then mixed with 50 cc. of water, expressed, mixed again with 50 cc. of water, and again expressed. The two aqueous extracts are mixed and filtered through filter-paper: the resulting yellowish filtrate has a very energetic action upon hydrogen peroxide (Sørensen).

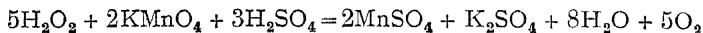
### Measurement of the Action of Catalase

452. In a flask are mixed a known volume of a very dilute solution of liver catalase (*e.g.* 100 cc. of the solution

in § 451 diluted to 1 in 1000) with 100 cc. of neutral hydrogen peroxide (diluted to 2 vols. strength). It is left at a low temperature in the ice-chest, and every thirty minutes 25 cc. are removed, and the contained hydrogen peroxide titrated. Control experiments are performed with boiled samples of catalase. The time-functions of catalase action can be studied by this method, as also the activities of different samples of catalase.

### Titration of Hydrogen Peroxide

453. When a solution of potassium permanganate is added to a solution of hydrogen peroxide which has been previously acidified with sulphuric acid, decomposition with liberation of oxygen occurs. Half the total oxygen evolved comes from the peroxide and half from the permanganate, as seen in the following equation :



from which it may be seen that one molecule of hydrogen peroxide corresponds to 2/5 molecule of permanganate.

To carry out the estimation the hydrogen peroxide is first diluted so that its titre is about "one volume" of oxygen. Generally speaking, 10 cc. of commercial peroxide diluted to 100 cc. gives a suitable strength. Ten cubic centimetres of this dilution are placed in a beaker with 100 cc. of water and 2 cc. of concentrated sulphuric acid ; then a decinormal <sup>1</sup> solution of potassium permanganate is run in, with constant shaking, until a faint red colour persists. Each cubic centimetre of permanganate corresponds to .0017 gramme of hydrogen peroxide, or to .0008 gramme of active oxygen produced therefrom (Denigès).

<sup>1</sup> Decinormal potassium permanganate is prepared by dissolving 3.16 grammes of the pure salt in 200 cc. of warm water, allowing to cool, and making the volume up to 1 litre.



## APPENDIX

## Glycolytic Enzyme of the Blood

454. Blood contains normally small amounts of sugar ( $\cdot 5$  to 1 gramme per litre). If allowed to stand the sugar disappears in a few hours owing to the action of a special enzyme which probably transforms it into lactic acid ("glycolysis"). The action of this enzyme is completely inhibited by the addition of sodium fluoride to the blood; and it can consequently be easily studied by receiving the blood of an animal in two bottles, one containing sodium fluoride to the extent of 2 per cent of the contained blood, and the other containing  $\cdot 1$  per cent of sodium oxalate to prevent clotting. By estimating the amount of sugar present in the two vessels from time to time it will be found that the sugar content of the fluorized blood remains constant, while that of the oxalated blood undergoes progressive diminution with the progress of glycolysis.

## Estimation of Sugar in Blood

455. To estimate the quantity of sugar in blood, the blood must be received immediately it is shed in a wide-mouthed bottle containing  $\cdot 2$  gramme of finely powdered sodium fluoride for each 100 cc. of blood. Both clotting and glycolysis are thereby inhibited. Twenty-five cubic centimetres of this blood are then measured in a pipette, put into a test-glass, and 125 cc. of 96 per cent alcohol are added, drop by drop, with continuous stirring. After ten minutes it is either filtered by means of the filter-pump (§ 124) or centrifuged (§ 135). The residue is then treated with 25 cc. of 80 per cent alcohol, and again filtered or centrifuged. This procedure is repeated once more; the alcoholic liquids are then mixed, and evaporated *in vacuo* (§ 149), until the volume of the residual liquid is reduced to about 15 cc. This is then transferred to a graduated flask of 25 cc. capacity, and the flask in which it was contained is washed out with a few cubic centimetres of water; these washings are added to the contents of the 25 cc. flask. Next, a solution of mercuric sulphate (§ 120) is added, drop by drop, to decolorize the liquid. The whole is shaken, and then neutralized by the addition of 10 per cent soda solution added, drop by drop, and the total volume made up to 25 cc. with distilled water and thoroughly mixed.

The liquid is next filtered on a little dry filter, and the colourless filtrate shaken up with a pinch of zinc dust, the precautions indicated in § 120 being duly observed. When all the mercury is removed the clear liquid is decanted and exactly 20 cc. are taken, in which the glucose content is estimated (§§ 105 *et seq.*).

The figure obtained is multiplied by 50, so as to express the result as grammes per litre of blood.

## CHAPTER XX

### ELEMENTS OF MICRO-BIOLOGY

#### General Principles

456. To study the chemical changes produced by various micro-organisms it is essential to work with pure cultures of typical character. The various species are isolated by the usual bacteriological methods; their purity controlled by their appearance when cultivated on suitable media, by microscopic appearances and by staining reactions.

When a pure culture has been successfully isolated it is allowed to act upon a specimen of the substance whose transformations are under investigation; this being dissolved in some suitable medium of as definite a character as possible. Such media, needless to say, must not contain substances which would interfere with the detection and estimation of the products of the reaction.

We are thus led in the study of fermentative processes to the consideration of culture media, inoculation and separation of micro-organisms, staining reactions, and the peculiar characters of each special fermentation.

In this connection it will be convenient to consider anaerobes separately, since they require special methods for their investigation.

#### Culture Media

457. Culture media may be either liquid or solid. Liquid media consist of solutions containing small amounts

of salts (such as sodium chloride, phosphates, etc.) and of organic substances (such as sugars, proteins and their derivatives, and other "nutrient" bodies). Meat-broth is often used, also decoctions of yeast or malt, with or without the addition of other materials such as glycerol, sugar, peptone, etc. Usually, cultures in liquid media are kept in ordinary test-tubes, each containing 10-15 cc. of the culture medium, which is conveniently run in from a funnel holding from 250 to 500 cc. The beak of the funnel is fitted to a rubber tube of a few centimetres in length, to which, at the other end, is fitted a glass tube drawn out to a convenient diameter at its free extremity. A pinch-cock is fitted to the rubber tubing. When sufficient of the culture medium has been run into a series of test-tubes, these are plugged with cotton-wool and sterilized as described in § 459.

458. Solid culture media are obtained by adding to liquid media, such as those previously described, varying amounts of gelatine or agar. These are liquefied by heat; from 10 to 15 per cent of gelatine and 1.5 per cent of agar being the proportions usually employed. Before adding these solidifying agents the liquid should be neutralized, as an excess of acid may hinder their setting to a suitable consistency. These media may be rendered clear by allowing them to cool to 40°, adding the white of an egg and again boiling. If coagulation of the added albumin is incomplete, a little acetic acid may be added, drop by drop, until the mixture is *very faintly* acid to litmus. When it is clear it should be neutralized again, or in some cases rendered faintly alkaline by the careful addition of soda. The medium, while still hot, is distributed in a series of test-tubes by means of the funnel previously described; the tubes are then plugged with cotton-wool and sterilized.

When a large surface is desired, only about 5 cc. of the medium is put into each test-tube. After sterilization the tubes are allowed to cool in a nearly horizontal position, so as to produce agar or gelatine "slopes."

Other solid media include egg-white and blood-serum

coagulated by heat, or slices of vegetable such as potato or carrot.

Solid media require protection from becoming dry owing to evaporation. This is effected by covering the mouths of the plugged tubes with little sterile rubber caps.

The composition of the medium selected for any particular purpose is of course regulated by the needs of the organism under consideration.

### Sterilization by the Autoclave

459. These different media, whether solid or liquid, are freed from living organisms, or "sterilized," by various means. Heating in steam under pressure is the most usual method. Exposure to a temperature of  $110^{\circ}$  for 30 minutes, of  $115^{\circ}$  for 20 minutes, or of  $120^{\circ}$  for 15 minutes, is sufficient for the sterilization of any medium. This heating is done in an autoclave, which consists of a strong metal vessel furnished with pressure-gauge and safety-valve, the lid of which is fastened down by suitable screws. This vessel contains a small amount of water, and the objects to be sterilized are placed in a suitable wire basket above the level of the water.

To use the autoclave sufficient water is put into the apparatus to reach nearly to the bottom of the wire basket, in which are then placed the articles to be sterilized; vessels containing culture media are plugged with cotton-wool, which is then covered with a double piece of paper secured with string. The lid is then put on the apparatus, and the screws fastened moderately lightly. The steam-tap is left open and the apparatus heated until all the air is expelled by steam. This can be recognized by the noise made by the escaping vapour, and also by the appearance of the issuing jet of steam. When the jet is composed of a mixture of air and steam the cloud produced by its condensation is continually changing in appearance, but when all the air has been expelled it presents a uniform opacity. The steam-tap is now closed and the hand on the pressure-

gauge kept under observation until it reaches the required figure.<sup>1</sup> When this is reached the temperature is kept constant by suitably regulating the flame.

After heating for a sufficient time (during which the gauge must be carefully watched), the light is turned off and the apparatus allowed to cool. When the hand of the gauge points to zero (*and not before*) the steam-escape tap is opened, the screws of the lid loosened, and the lid removed.

### Preparation and Sterilization of Pasteur's Pipettes

460. First cut off lengths of glass tubing measuring 20-25 cm. in length by means of a glass-cutter or file ; from 7 to 8 mm. is a convenient diameter. The two ends should be rounded off by heating them in the flame, and after cooling each end should be packed with cotton-wool for about 2 cm. of its length. The cotton-wool plug must not be allowed to project at the ends ; it is most conveniently pushed home with an iron wire or a piece of glass rod suitably drawn out. The tubes are then sterilized by heating in the hot air-oven to 180° for fifteen minutes. If the sterilization is efficient the woollen plugs will be slightly browned, or at least will have a yellow colour. After cooling the sterile tubes are kept until wanted for use.

They can also be sterilized in the autoclave at 120°, but they must be dried in the air-oven before use.

When a sterile pipette is required one of these tubes is heated at its centre by repeatedly rotating it between the fingers in the flame. When the glass has been sufficiently softened, it is withdrawn from the flame and drawn out, so as to leave a narrow portion measuring from 40 to 50 cm. ; the bore should not be made too fine. This thinned portion is cut so as to leave two pipettes, the ends of which are immediately sealed. When the pipette is required for use the sealed end is broken off.

<sup>1</sup> If a jet of steam issues from any point around the circumference of the lid the screws in that situation are slightly tightened.

### Inoculation of Culture Media

461. The object of this operation is to convey a pure culture of some given micro-organism to a suitable sterile culture medium without any contamination from extraneous sources. The culture is accordingly transferred either with a sterile pipette or with a platinum wire, first heated to redness and then allowed to cool, care being taken at the same time to avoid contamination from organisms present in the air.

With this object test-tubes or the necks of flasks are held in a sloping position when their plugs are removed, and their mouths are flamed before reintroducing the plug. The plugs must on no account be laid down during the manipulations, but must be held so as to avoid any possibility of contamination of the part which will be again placed in the test-tube or flask. If Pasteur pipettes are used for inoculation their ends must be flamed after breaking off the extremity; any possibility of contamination from this source is thereby avoided. The whole operation is conducted for preference in a perfectly still atmosphere so as to eliminate the possibility of contamination from floating particles of dust.

It is a good plan to cover the plugs, after the tube or flask has been inoculated, with a little cap made of a double layer of filter-paper; this can be kept in place by a piece of thread if necessary.

### Separation and Isolation of Organisms

462. The object of the following procedures is to obtain pure cultures of the micro-organisms under investigation. They may be based either upon mechanical principles or upon some physiological character of the organisms, such as their resistance to heat, for instance.

Mechanically, organisms can be separated in the following manner: A platinum wire of sufficient thickness mounted in a piece of glass rod, or simply a glass rod appropriately

drawn out, is sterilized in the flame, and allowed to cool ; a small portion of the growth under examination is removed, and spread over the surface of (*e.g.*) an agar slope. The wire is passed to the bottom of the tube and drawn up over the contained medium in a zig-zag manner, so as to spread the organisms over the surface of the medium. A second and third tube are inoculated in the same way, with the same rod, and without taking up a fresh supply of the original culture. The mouths of the tubes are then flamed and plugged. The rod is thus gradually freed from the adherent micro-organisms and is flamed again before being laid down. The organisms being spread over the surfaces of the nutrient media, when these are placed in the 35° incubator for a suitable period, develop into more or less isolated colonies. Such a colony is removed by the platinum wire as before and inoculated into a suitable sterile medium.

Before proceeding to this final series of inoculations the colonies should be examined microscopically to determine their purity, since colonies, though of homogeneous appearance, may consist of two or more associated organisms.

Another method of inoculation is to dip the end of the platinum wire conveying the organisms into the condensation water in each of four successive tubes (without recharging the wire), and stirring it somewhat so as to disperse the organisms. The inoculated water is then allowed to flow over the surface of the nutrient medium by sufficiently inclining the tubes, which are then placed in the incubator.

463. A second method of isolation depends upon the resistance to heat of some given species or of its spores. This may be utilized, for instance, in the case of *Bacillus subtilis*. The spores of this species, which is widely distributed on the surface of the soil and on fodder, resist the action of a temperature of 100° for some time ; this temperature is however sufficient to destroy the organisms in their vegetative stage. A pure culture of *B. subtilis* may be obtained by the following procedure :



A little dried hay is infused for half an hour in an evaporating basin with sufficient warm water to cover it. The liquid is filtered into a flask, which is then plugged with cotton-wool and boiled for at least a quarter of an hour. It is then allowed to cool and placed in the 35° incubator. After an incubation of from 24 to 48 hours the surface of the liquid will be covered with a thin layer of growth of *B. subtilis*. A fragment of the film should be examined microscopically, and also a drop of the liquid which contains

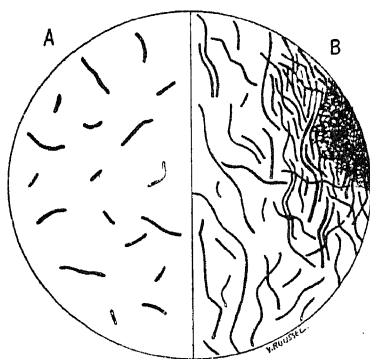


FIG. 43.—*Bacillus Subtilis*.

A. Young culture. B. Edge of pellicle.

motile bacilli joined end to end, forming filaments of greater or less length (Fig. 43).

### Isolation of Anaerobes

464. The isolation of anaerobes is performed in the following manner. (Veillon and Zuber): In the first place, 250 grammes of fat-free meat are minced and macerated in 500 cc. of water. This is left for two or three days, strained through muslin, and 5 grammes of peptone and 2.5 grammes of sodium chloride are added to the liquid. The mixture is slightly heated and stirred thoroughly; when solution is complete 6 grammes of agar, cut into small fragments, and enough 10 per cent soda solution to make

the liquid definitely alkaline to litmus-paper are added. It is then autoclaved at  $120^{\circ}$  for half an hour, and allowed to cool at  $50^{\circ}$ , when half the white of an egg, beaten up with a little water containing 7-8 grammes of glucose, is added. The mixture is again thoroughly stirred and kept at  $120^{\circ}$  for fifteen minutes; whilst still boiling it is filtered through Chardin's paper. It should filter easily and yield a clear filtrate. The medium is then distributed in test-tubes as described in § 457; each test-tube should be filled to a height of about 10 cm. The test-tubes are plugged with cotton-wool and sterilized at  $115^{\circ}$  for fifteen minutes.

465. To isolate anaerobes contained in the soil, for example, a small pinch of garden earth is mixed with 10-15 cc. of water and then allowed to settle. At the same time five or six test-tubes containing the medium described in § 464 are placed in boiling water so as to melt the agar, withdrawn after five or six minutes and kept at  $40^{\circ}$  in the water-bath; at this temperature (which is harmless to the organisms) the agar remains liquid.

The tubes are inoculated in the following manner: A sterile Pasteur pipette, finely drawn out and closed at the end, is dipped into the soil-washings so as to remove a drop of the water. This is introduced into the first tube and stirred so as to distribute any organisms present. The pipette is stirred in the other tubes in succession (without being again dipped in the washings), the operations being conducted with all the usual precautions against contamination.

Thus a series of tubes is obtained containing increasing dilutions of the organisms, and wherein they are progressively fewer and more sparsely disseminated. The tubes are then stood in cold water until the medium sets and the organisms thereby maintained in place. They are then transferred to the  $35^{\circ}$ - $37^{\circ}$  incubator.

If the tubes have been well freed from air by heating to  $100^{\circ}$  the contained media will retain only very little oxygen, and although penetration occurs from the surface it only occurs for a depth of 1-2 cm. In this zone, therefore, aerobes will grow; while the anaerobes develop in the

deep parts of the medium and gradually form colonies of varying appearance.

These colonies, although very numerous in the first tubes, should be fewer and well separated in the last. They can easily be examined either with a good hand lens or under a low power of the microscope.

To isolate any particular colony its position is marked on the surface of the tube by an ink spot. A fine Pasteur pipette (with the point broken off) is sterilized in the flame and thrust through the agar until the desired colony is reached. By means of slight suction through a rubber tube attached to the pipette the organisms are drawn up. The pipette is then withdrawn, care being taken to avoid contact with any neighbouring colonies, and the selected medium inoculated by blowing slightly through the pipette. The majority of anaerobes, especially when sugar is present, produce gas, which breaks up the solid medium. In the majority of cases this gas formation may be inhibited by the addition of .1 per cent of potassium nitrate to the medium.

### Fixing and Staining of Micro-Organisms

466. To obtain the natural appearances of bacteria they must be examined in the living state, when their form, size, and motility may also be determined. The examination is easily made by putting a drop of a liquid culture on a slide, covering with a cover-slip and examining the preparation microscopically. If the culture is on a solid medium a minute fragment of the growth is removed, rubbed up on the slide with a drop of water, covered, and examined as before. The light must be carefully regulated for the examination of living organisms; too much light may completely prevent their being seen.

Excellent results are also obtained by examination with the dark background.

To examine cultures of anaerobes, the preparation is covered as before, but special care is taken to avoid the

inclusion of air-bubbles. A drop of melted paraffin-wax is placed at each of the four corners of the cover-slip. By means of a warm, curved, iron wire the paraffin is made to run along the edges of the cover-glass so as to seal the preparation. Motile anaerobes at first lose their motility, which is however gradually regained.

467. When it is desired to detect bacteria in tissues, or to examine their structural details, it is necessary to stain them. The procedure is as follows: A slide is first cleaned with a fine linen rag moistened with a little ammonia. A drop of the culture (or watery suspension in the case of bacteria grown in solid media) is spread out to make as thin a film as possible and occupying 1-2 sq. cm. of surface. This is allowed to dry in the air. When dry it must be "fixed." By this process the organisms are made to preserve their form and to adhere to the slide during subsequent manipulations. A mixture of equal parts of alcohol and ether is allowed to flood the preparation; this is poured off and the operation repeated. The specimen is then allowed to dry in the air. For staining, various reagents may be used, such as a saturated watery solution of methylene blue, fuchsin, or gentian violet. These solutions must be filtered immediately before use. After a minute or two excess of stain is poured off, the specimen washed with water and allowed to dry. If the staining should prove insufficient the operation is repeated, the stain being allowed to act for a little longer period.

### Double Staining

468. Under certain circumstances it is advantageous to have a double stain; for example, in the demonstration of nuclei or spores, or in the differentiation of bacterial species in a mixture. It is not proposed here to detail the almost innumerable methods of double staining employed in bacteriology, but simply to exhibit the principles of the process by means of a few simple examples. Put generally, it may be said that the whole of the fixed preparation is

stained with some appropriate dye and then treated with a differentiating agent. By placing the preparation in a second stain, this will only colour the portions which have been decolorized. Such colours are chosen as will ensure a good contrast; violet and red, blue and red, red and green, etc.

469. A very simple example of double staining is the case of cell nuclei. We know that the nuclear chromatin strongly fixes "basic" dyes such as methylene blue, fuchsin, etc. If the preparation is first treated with an "acid" dye—eosin, for example—the whole will be diffusely stained. On now using a basic dye, this displaces the other in the cell nuclei which are stained blue. By this method double staining is effected without special differentiation or decolorization.

The nucleated red corpuscles of amphibia, reptiles or birds make excellent preparations by this method. Birds (for example pigeons) may be bled by puncturing a large vein which runs along the edge of the wing; the exuding drop of blood is taken up on a *clean* glass slide, and spread out in as thin a film as possible by means of a thin card or another microscopic slide. The preparation may be rapidly dried by waving it to and fro in the air. It is fixed by means of absolute alcohol which is allowed to act for five or six minutes; two lots of alcohol should be used. It is then flooded with a .5 per cent watery solution of eosin for about a minute; this is then poured off and replaced by a saturated watery solution of methylene blue which is allowed to act for about thirty seconds. This is poured off and the specimen washed in distilled water; as long as the washings are coloured it continues to bring colour away. The specimen is then dried and examined under the microscope. The nuclei of the red cells will be stained blue while the surrounding protoplasm is pink.

470. We may take the staining of spores as a second example. Bacterial spores are, generally speaking, difficult to stain, and require preliminary treatment by some suitable mordant. When stained, however, the stain is equally

difficult to remove; decolorizing agents do not affect it, so that upon treating the specimen with a second stain, this will only affect the bodies of the bacteria, the spores retaining the first colour. The double staining effect is produced in the following manner. A portion of a 24-48 hours' old culture of *B. subtilis* which contains sporing forms (§ 463), or of a two or three days' old culture of the butyric acid ferments from soil (§ 486), supplies suitable material for this purpose.

A drop of the culture, or a fragment of the film suspended in a drop of water, is spread out on a slide, allowed to dry and fixed by alcohol-ether for two or three minutes. A few drops of a 5 per cent solution of chromic acid are next allowed to act for five minutes; this serves as the mordant. The chromic acid is washed off with distilled water and a sufficiency of carbol-fuchsin<sup>1</sup> to cover the preparation is poured on. This is warmed gently over a small flame until steam arises, fresh quantities being added from time to time, *as the specimen must not be allowed to become dry*. The heating is continued for five or six minutes. Excess of stain is removed by thorough washing in water, and differentiation is effected by immersing the slide in a 2 per cent aqueous solution of aniline hydrochloride. The specimen is next washed in 96 per cent alcohol which decolorizes everything except the spores. It is then examined roughly under the microscope to ensure that decoloration is complete; if it is incomplete further treatment with aniline hydrochloride and alcohol is essential. A saturated aqueous solution of methylene blue is next allowed to act for about a minute; this stains the bacteria blue. The slide is then washed in water, allowed to dry, and examined under the microscope. Bacteria then are seen to be stained blue and spores red.

471. Finally we may consider the process of double staining which is known as "Gram's method." When a

<sup>1</sup> This solution ("Ziehl's fuchsin") is prepared by mixing 10 cc. of a 10 per cent alcoholic solution of fuchsin with 90 cc. of 1-2 per cent solution of ordinary phenol in water. It must be filtered before use.

preparation is stained with one of the pararosaniline derivatives, then treated with a solution of iodine in potassium iodide and finally with absolute alcohol, it will be found that in some cases the stain is retained by the organisms ("Gram-positive" organisms), while in other cases it is removed by the alcohol ("Gram-negative" organisms).

This method is largely used in Bacteriology, since it affords a ready means of differentiating many species.

When dealing with a mixture of organisms, some of which are "Gram positive" (Ordinary yeast; *Saccharomyces vini*; *B. subtilis*, etc.), while others are "Gram negative" (Acetic-acid-ferment, etc.); after staining by Gram's method and decolorizing in alcohol, the Gram-negative organisms can be stained by some contrast stain, thus giving a sharp differentiation between the different organisms.

The mixture of acetic-acid-ferment and *Saccharomyces vini* which occurs in vinegar when it is exposed to the air, will give an admirable specimen to demonstrate this method of staining. For this purpose a fragment of the film which contains these organisms is spread out in a drop of water on a clean slide, allowed to dry and fixed in absolute alcohol for two or three minutes. It is then stained in aniline gentian violet<sup>1</sup> for 3 or 4 minutes. The excess of stain is tilted off, and without washing the specimen is treated with Gram's iodine solution (1 gram of iodine, 2 grammes of potassium iodide and 300 cc. of water) for about two minutes. The preparation, which is of a brown colour, is then washed with absolute alcohol until no more colour comes away; it is then dried and examined microscopically, when the yeast will be found to be stained a deep violet while the acetic-acid-ferment is decolorized. This latter

<sup>1</sup> To prepare this reagent, which should be made as required, to 10 cc. of a saturated aqueous solution of aniline, is added about 1 cc. of a saturated alcoholic solution of gentian violet. This is enough to make a deep opaque violet colour. The presence of a sufficiency of the stain is assured by its forming a fine superficial iridescent layer.

Commercial specimens of gentian violet always contain a certain amount of dextrin which is insoluble in alcohol. Hence the alcoholic solution must always be filtered immediately before use.

may be stained red with a saturated watery solution of fuchsin allowed to act for 20 or 30 seconds. The specimen is then washed in water, allowed to dry and examined.

472. The original method of Gram has been modified by Nicolle in the following manner. A solution of carbol-gentian violet (prepared in the same way as Ziehl's carbol-fuchsin, § 470) is allowed to act for five or six seconds only; it is replaced by iodine solution (iodine 1 gramme, potassium iodide 2 grammes, water 200 cc.), which is renewed two or three times and left in contact with the specimen for 5 or 6 seconds. The specimen is then washed with a mixture of five parts absolute alcohol with one of acetone, poured on drop by drop as long as stain comes away. It is then washed in water, dried and examined microscopically.

Nicolle's method is more rapid, but perhaps requires a little more delicacy of manipulation than Gram's original method.

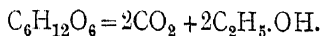


## CHAPTER XXI

### THE PRINCIPAL FERMENTATIONS

#### Alcoholic Fermentation

473. THE most favourable media for the development of yeast contain sugar with suitable nitrogenous and mineral constituents, and are of neutral or feebly acid reaction. Yeast causes sugar to undergo alcoholic fermentation according to the following equation :



Saccharose is first hydrolysed by the sucrase of the yeast, while maltose is hydrolysed by maltase. The alcohol and carbon-dioxide produced as the result of fermentation are accompanied by small amounts of glycerol and succinic acid.

474. The following culture medium is recommended for yeast :

Yeast water (or malt water)	200 cc.
Saccharose . . . . .	20 grams.

The sugar is dissolved and the mixture autoclaved at  $115^\circ$  for fifteen minutes.

Yeast water is prepared by boiling 1 part of compressed commercial yeast with 20 parts of water for ten minutes and then filtering. Malt water is made by boiling the dried radicles of the germinating grain (residue of malt) in water and filtering, the proportions being the same as in the case of yeast. This latter medium is more conveniently prepared

by making a solution of malt extract (commercially sold as "maltopeptone") of a strength of 5-10 grammes per litre of water.

475. To obtain yeast in pure culture it is sufficient to inoculate such a medium with the deposit from the bottom of a bottle of wine or from a recently emptied bottle of beer. It is left in the 25° incubator, and at the end of three or four days alcoholic fermentation will be in full progress. The yeast can then be isolated by inoculating agar slopes, prepared with yeast or malt water with the addition of 1.5 per cent of agar.

Yeast colonies appear as round, white and shining, and give the appearance of droplets of cream deposited on the surface of the agar. One such colony should be picked off and inoculated (with the precaution mentioned in § 46r) into a tube of yeast-water. This is incubated at 25°; carbon-dioxide is evolved, the sugar almost entirely disappears, and the alcohol formed can be isolated and estimated.

When the cultures have been obtained from wine-dregs, in addition to ordinary yeast (Fig. 44) colonies of *Sac-*

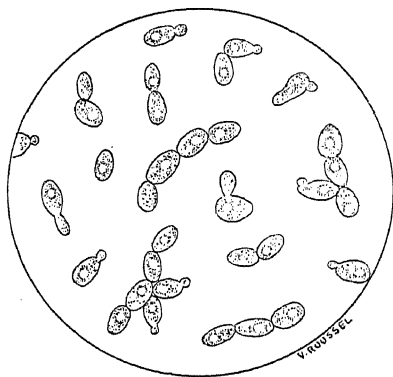


FIG. 44.—Ordinary Yeast (*Saccharomyces cerevisiae*).

*charomyces apiculatus* are also seen. These microscopically resemble colonies of ordinary yeast, but the individual cells

when examined microscopically show a characteristic outline not unlike that of a lemon (Fig. 45).

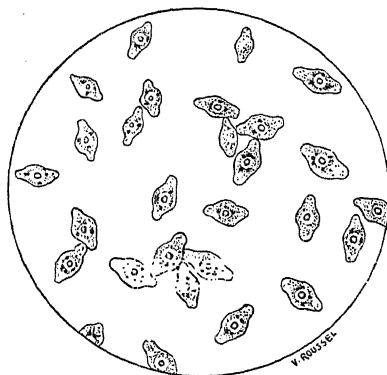
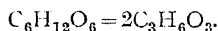


FIG. 45.—*Saccharomyces apiculatus*.

### Lactic Fermentation

476. Lactic ferments are numerous and widely diffused. They are found as rods or as cocci (in which latter case they often form chains) in the dust from fodder, and hence occur in cow-sheds where they contaminate the milk. At a temperature of from 30° to 35° they energetically act upon lactose, which is decomposed and then converted into lactic acid.



As a result the milk becomes markedly acid, and when about 1 per cent of acid is present clotting occurs ("spontaneous clotting" of milk).

477. To isolate the ordinary lactic ferment, 10 cc. of milk are placed in a test-tube in the 30° incubator. When clotting is complete, the tip of a sterile pipette is plunged beneath the surface of the supernatant cream, a drop of the liquid withdrawn and inoculated into a suitable liquid medium. Such a medium is malt-water (§ 474) with glucose or lactose to which 5 per cent of calcium carbonate has been added. This combines with the lactic acid produced

by the fermentation and so removes its inhibitory effect upon the growth of the organisms.

A suitable medium for this purpose consists of—

Extract of malt (maltopeptone)	5 grammes.
Sugar (glucose or lactose) . . .	10 „
Calcium carbonate . . . . .	10 „
Water . . . . .	200 „

This is sterilized at  $115^{\circ}$  for twenty minutes, and after cooling it is inoculated. The sugar rapidly disappears at  $30^{\circ}$ - $35^{\circ}$ , and there is abundant production of lactic acid. In order to isolate the lactic ferment in a pure condition,

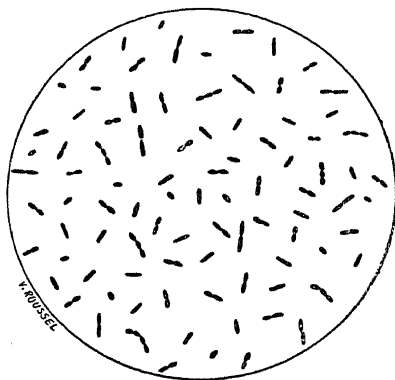


FIG. 46.—Lactic Acid Ferment (*Bacillus acidilactici*).

cultures are made upon agar slopes made up with the above medium. The lactic ferment forms transparent colonies which look like droplets of water. Microscopically it appears as short rods, generally joined together in groups of two or three (Fig. 46).

### Acetic Fermentation

478. Wine when exposed to the air may easily become acid. Under these circumstances a very thin, fragile, and slightly iridescent film makes its appearance upon the surface. Microscopically this is seen to consist of very

short rods arranged parallel to one another. In many cases, however, instead of this thin growth of *Mycoderma aceti*, a thicker dead white layer with crinkled edges is seen, due

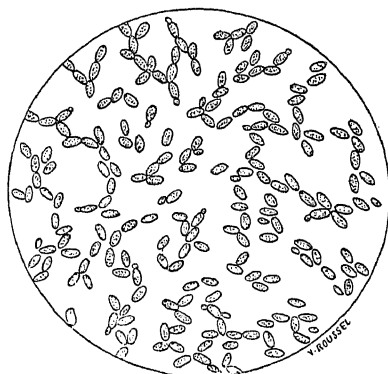


FIG. 47.—*Saccharomyces vini*.

to the growth of *Saccharomyces vini* ("fleur de vin"). The individual elements of this growth are much larger than those of the acetic acid ferment, and resemble yeast cells

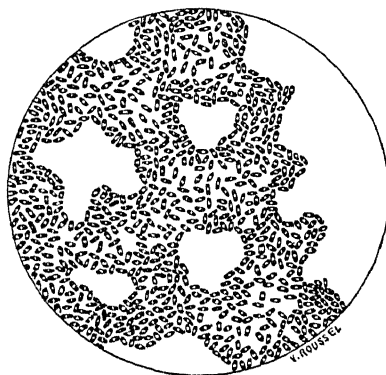


FIG. 48.—Pellicle of *Mycoderma aceti*.

both in size and in their method of propagation by budding; they differ from the cells of ordinary yeast, however, in their more elongated outline (Fig. 47). Very often this

growth makes its appearance before *Mycoderma aceti*; but in this case the *saccharomyces* disappears gradually and gives place to the characteristic growth of *Mycoderma aceti* (Fig. 48).

The acetic ferment is generally transported by a little reddish fly (*Drosophila cellaris*) which comes to the alcoholic liquid in order to lay its eggs.

479. In place of pure wine the following medium may be used for obtaining the acetic ferment :

Wine (red or white)	1 part.
Water . . .	2 parts.
Wine vinegar <sup>1</sup> .	1 part.

This mixture is placed in a wide, open glass dish to a height of from 4.5 cm., and is left at a temperature of about 28°.

480. To observe the phenomena of acetic fermentation a portion of the thin layer, which either appears in the first instance or after the disappearance of the growth of *Saccharomyces vini*, is removed with a sterile rod and inoculated into a sterile medium disposed in a thin layer in a conical flask (50 cc. of liquid in a flask of 250 cc. capacity). The culture is placed in the 28°-29° incubator, and after a day or two the film will be found to have extended all over the surface. The alcohol—which is oxidised to acetic acid—rapidly disappears. The change may be demonstrated quantitatively according to the instructions in §§ 183 and 497.

481. The most suitable medium for the cultivation of the acetic acid ferment is yeast-water to which alcohol has been added. To prepare this, 10 grammes of commercial compressed yeast are mixed with 10-20 cc. of water, and the suspension is added to 80-90 cc. of water heated in a porcelain capsule. It is brought to boiling and kept boiling for five minutes, being stirred the whole time. It is then filtered through Chardin's paper and allowed to cool to

<sup>1</sup> As wine vinegar often contains "sorbosc bacteria," it should be heated to nearly boiling for a minute in order to sterilize it.

30°-40°, when a little egg-white is added. The mixture is then autoclaved at 115° for ten minutes, and again filtered. The filtrate should be diluted with water so as to contain .5 per cent of extract<sup>1</sup>, and 5 per cent of alcohol is added. The medium is then distributed in a series of conical flasks of 250 cc. capacity, each containing 50 cc. of the liquid; these are then autoclaved at 110° for 20 minutes. The resulting loss of alcohol is very small.

### Oxidation of Glycerol by Sorbose Bacteria

482. This bacillus acts upon glycerol so as to convert it into a ketone sugar, dioxycetone,  $\text{CH}_2\text{OH} \cdot \text{CO} \cdot \text{CH}_2\text{OH}$ .

To obtain the organism, a mixture of equal parts of wine, water and (unheated) vinegar is kept either at the room temperature or at 30° in the incubator. In a few days, translucent, slightly raised spots with a shining surface make their appearance, and when they have attained a sufficient degree of development resemble spots of suet. These spots are colonies of sorbose bacteria; the organism is either transmitted in the sample of vinegar used or by small flies from vinegar factories. Frequently, as in the case of the spontaneous development of *Mycoderma aceti*, a growth of *Saccharomyces vini* precedes or accompanies the appearance of these bacteria.

To isolate the organism, prepare yeast-water containing 5 per cent of extract (see § 481), add to it 2-3 per cent of glycerol and 1.5 per cent of agar, and prepare "slopes." These are then inoculated from suitable colonies grown as described above.

483. When a pure colony of the bacillus has been obtained it is inoculated into yeast-water containing 3 per cent of glycerol, which is placed in conical flasks of convenient size to a depth of 4-5 cm. and sterilized in the autoclave before inoculation. The culture when left at 28°-29° gradually forms a thick, whitish, translucent mass,

<sup>1</sup> Ten cubic centimetres should be evaporated to dryness in a porcelain capsule in order to ascertain the percentage of extract present.

which is seen microscopically to consist of a mass of rod-like organisms (Fig. 49). In a few days the liquid will be found to have acquired marked reducing properties

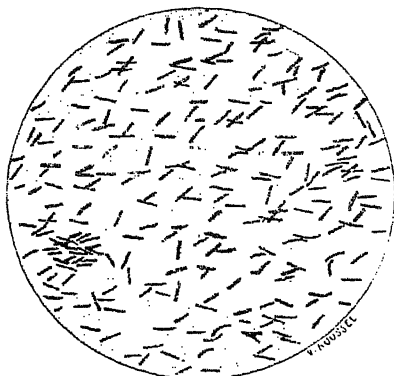
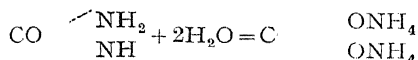


FIG. 49.—Sorbose Bacteria.

in consequence of the presence of dioxyacetone. This substance may be recognized from its almost immediate reduction of Fehling's solution *in the cold*.

### Ammoniacal Fermentation

484. Many micro-organisms have the power of producing ammonium carbonate from urea by hydration.



An impure culture of urea ferments may be obtained by exposing to the air a beaker half filled with urine at a temperature of 25°-30°. At the end of from 24 to 48 hours the liquid will have become turbid and definitely alkaline in reaction, as a consequence of the formation of ammonium carbonate. If the fermentation has been sufficiently active a marked odour of ammonia will be perceptible.

485. The organisms can be isolated in pure culture by inoculation of broth-agar slopes (§ 464), to which 1 per cent of urea has been added.



It is, however, better to use a medium of meat broth (§ 464), containing 10-12 per cent of gelatine and 1 per cent of urea. Slopes of this medium are inoculated in the usual manner. When colonies of the urea-fermenting organisms develop on this medium they have a characteristic whitish halo surrounding them; this halo is due to the precipitation of phosphates of the alkali-earths contained in the gelatine, by ammonia formed by the activity of the organisms.

Such a colony is inoculated into a specimen of urine previously sterilized by heating to  $110^{\circ}$  for a quarter of an

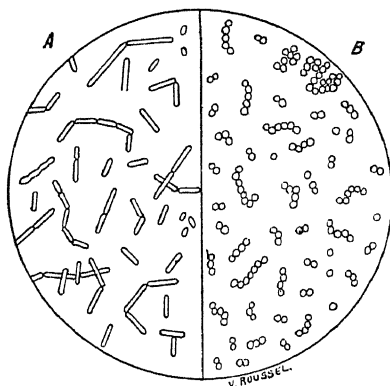


FIG. 50.—Urea Ferments.

A, Bacilli. B, Cocci.

hour. By this means either bacilli (which are often joined together) or cocci are obtained (Fig. 50). The progress of the fermentation can be followed by removing 5 cc. of the liquid from time to time and estimating the ammonia as in § 533.

### Butyric Fermentation

486. Existing in the soil are numerous anaerobic organisms capable of setting up butyric fermentation in starch, sugars, etc. Glucose, for example, undergoes the

change indicated by the following equation:  $C_4H_{12}O_4 = C_4H_8O_2 + 2CO_2 + 2H_2$  being decomposed into butyric acid, carbon dioxide, and hydrogen.

A good sample of butyric ferment can be obtained by leaving some slices of unwashed potato in a test-glass filled with water and kept at  $40^\circ$ . After from 24 to 48 hours the surface of the liquid will be covered with froth and a strong smell of butyric acid will be noticed. At the same time the potato slices will show signs of decomposition.

If a drop of the liquid is put on a slide, covered with a large coverslip and examined microscopically, unchanged

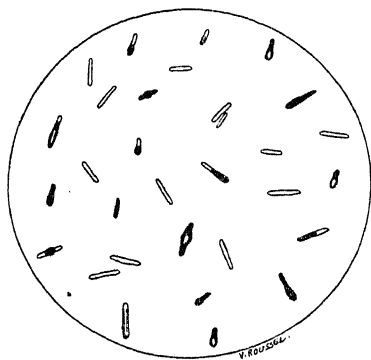


FIG. 51.—Butyric Acid Ferment.

starch grains are seen around which are numerous micro-organisms, either of rod or spindle shape. As long as these bacteria are in the centre of the preparation they show active movement, but at the edges where they are in contact with atmospheric oxygen they are non-motile. By running a drop of iodine solution under the cover-glass most of the rods will be stained blue; this reaction suggests that the organisms contain starch. In older cultures where only sporing forms are seen no blueing occurs on the addition of iodine.

487. To obtain a pure culture of butyric ferment, the technique described in § 465 should be followed and a colony inoculated into the following medium :

Saccharose . . . . .	6 grammes.
Ammonium phosphate . . . . .	·1 to ·2 gramme.
Calcium carbonate . . . . .	5 grammes.
Water . . . . .	200 cc.

Butyric fermentation of saccharose can be obtained by inoculating some of this medium with the crude growth obtained from potato (§ 486), but the flask must be filled to the neck with the medium so as to avoid the influence of oxygen. The temperature should be kept at about 40°.

488. To collect the gases given off during fermentation the fermentation flask should be furnished with a cork carrying a bent glass tube, with the free extremity plunged below the surface of a basin of water. When fermentation is active the gases can be collected in an inverted test-tube filled with water and examined. As the equation in § 486 indicates these gases consist of carbon dioxide and hydrogen, as may be shown by the following experiment: Introduce a potash pastille, close the test-tube with the thumb and shake, the carbon dioxide will be absorbed, and by removing the thumb under water, the water will be seen to rise in the tube. When all the carbon dioxide is absorbed it will be found that the residual gas is inflammable when a light is applied to it and burns with a pale flame. The liquid in which the fermentation has occurred contains a mixture of volatile acids, among which butyric acid is present in the largest quantity. It can be detected and estimated by Duclaux's method (§ 521).

## APPENDIX

### SEPARATION OF ENZYME AND MICROBIAL ACTIONS

#### Filtration with the Filter-Candle

489. In the fermentations just considered we have not been specially concerned with the precise mechanism of the active agent. The chemical changes brought about in substances specially introduced into certain culture media may have been

effected either by extracellular enzymes or by the direct action of the living protoplasm itself. Frequently a given change will result from the action of a series of enzymes followed by action of the living substance of the cells themselves. To differentiate between these two classes of phenomena a mechanical means of separation may be adopted, such as centrifuging or filtration. By this means a liquid can be obtained which is active if the observed processes are due to extracellular enzymes, but which is inactive if due to the living protoplasm of the organisms itself. In some cases enzymes are so closely linked with the protoplasm of the organisms which produce them, that these have to be disintegrated by more or less elaborate

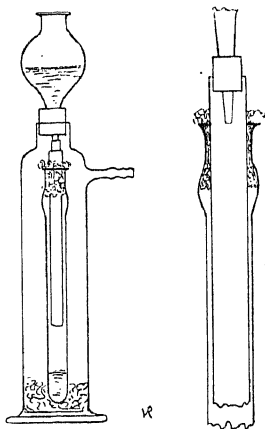


FIG. 52.—Filtration by Filter-candle.

methods of crushing before the enzymes can be separated either by the centrifuge or the filter.

The most generally used method for the separation of enzymes from the micro-organisms producing them is filtration through a porous earthenware candle, which not only separates enzymes from cells but leaves the filtrate sterile, so that the enzyme-action can be studied under conditions of rigorous asepsis.

490. The technique is very simple. First ascertain that there are no flaws in the filter-candle, by plunging it into water with its closed end downwards, then blow in air under slight pressure with a bicycle pump. If there is a localized formation of air bubbles at any particular point, this indicates a flaw, and the candle must be rejected. If the apparatus is intact it is

fixed in the glass cylinder of the apparatus shown in Fig. 52, by means of a plug of cotton-wool. This cylinder must contain a few cubic centimetres of water. The funnel is adjusted to the filter-candle by means of a small, perforated, rubber cork, and again the inside of the candle and funnel must be moistened. The mouth of the funnel is now closed with a cotton-wool plug. The whole apparatus is sterilized in the autoclave for from fifteen to twenty minutes.

In order that the sterilization may be effective the inside of the apparatus must be full of steam, and when withdrawn from the autoclave the glass cylinder must always show the presence of a little water.

The whole apparatus is now placed in the tabulated cylinder and kept in place by a cotton-wool plug at the bottom and by the well-fitting rubber cork at the top. The liquid to be sterilized is put in the funnel, the outer glass cylinder is connected with the filter-pump, when the liquid filters through the candle and is collected in the surrounding sterile glass tube. When the operation is complete, portions of the liquid may be removed with a sterile pipette under all usual aseptic precautions.

491. This method may be used for studying the enzymes produced by *Aspergillus niger*. When this fungus has finished its development on Raulin's solution (after about three or four days) the liquid is removed and replaced by distilled water. This is done two or three times so as to wash the lower surface of the mycelium, which is finally floated out on a new quantity of distilled water. The culture is replaced in the 35°-37° incubator for twenty-four hours.

At the end of this time it can be shown that the water in which the mycelium is undergoing maceration has developed considerable enzyme activity, especially towards saccharose (Gayon). But besides sucrase it contains maltase, emulsin, amylase, proteoclastic enzymes, etc. These various enzymes, however, on account of their small activity, require some considerable time for its manifestation. Hence the necessity for their study under aseptic conditions. Sterile solutions of maltose, glucosides, etc., are prepared, and to these is added the watery extract of *Aspergillus niger*, sterilized by passage through a filter-candle.

It should be noted that if this liquid in which *Aspergillus* is macerated has an acid reaction, the enzymes are very largely held back by the filter, so that it is desirable to neutralize before filtering.

## CHAPTER XXII

### IDENTIFICATION AND ESTIMATION OF THE CHIEF PRODUCTS OF FERMENTATION

#### Detection of Alcohol

492. SMALL quantities of alcohol are produced in many fermentative processes other than the typical alcoholic fermentation. To test for this substance, the liquid under examination is placed in a flask, or if the material is solid it is broken up, mixed with water, and similarly treated. The flask is connected with a glass condenser (preferably Schloesing's apparatus). Distillation is started, and allowed to continue until about  $\frac{1}{10}$ th of the total volume of liquid has passed over. This portion of the distillate will contain all the alcohol originally present in the mixture. The wall of the condensing tube should be carefully watched at the commencement of the operation; if alcohol be present, even in traces, droplets and oily-looking streaks will be noticed. This appearance is not characteristic of alcohol alone, but merely indicates the presence of a volatile liquid mixed with steam.

If a sufficient quantity of distillate is available it may be redistilled so as to free the alcohol as much from water as possible and concentrate it in a small volume of water.

493. To show the presence of alcohol, take in a test-tube about 1 cc. of the distillate obtained as above, add a drop of benzoyl chloride, and then gradually with constant shaking a few drops of 10 per cent soda. When the droplets of the reagent have disappeared, close the mouth of the tube with the thumb and shake so as thoroughly to moisten

the sides of the tube ; on removing the thumb the characteristic odour of ethyl benzoate will be perceived. A 1 per cent solution of alcohol will give this reaction with perfect distinctness.

494. To another cubic centimetre of distillate add 3 cc. of concentrated nitric acid containing in solution .5 per cent of potassium bichromate. In a few minutes a blue violet colour appears (in the cold) if the liquid contains a body containing an alcohol radicle. This reaction is also given by aldehydes but not by ketones (H. Agulhon).

495. *Formation of Iodoform.*—To 2 cc. of distillate in a test-tube are added one or two drops of soda solution, and

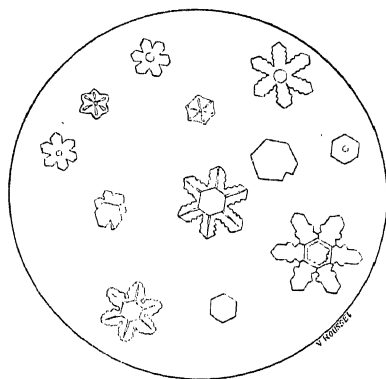


FIG. 53.—Crystals of Iodoform (Microscopic).

the mixture is gently warmed to  $45^{\circ}$ - $50^{\circ}$  ; a 1 per cent solution of iodine (§ 132) is then added, until it is distinctly yellow. Allow it to cool very slowly and remove with a pipette a small quantity of the yellow deposit which is formed. This when examined microscopically will show the characteristic yellow crystals of iodoform, recognizable by their symmetrical hexagonal shape (Fig. 53). It must not be forgotten that many other substances—among them aldehyde and acetone—also give this reaction. But aldehyde reduces ammoniacal silver nitrate solution, and acetone does not give the characteristic blue colour with the nitric acid and potassium bichromate reagent (§ 494).

496. If the quantity of alcohol contained in the distillate is not too small it can be separated by saturating the liquid with dry powdered potassium carbonate. The alcohol forms a supernatant layer which can be removed by a pipette and recognized by its smell, inflammability, and other physical and chemical properties.

### Estimation of Small Quantities of Alcohol

497. The alcohol is first separated and concentrated as an aqueous solution (§ 492) by fractional distillation, but the exact quantities of the original liquid and the distillate must be carefully noted. Its amount can then be estimated either by chemical or physical methods.

498. *Physical Method: Drop - Counter.* — Mixtures of alcohol and water have a lower surface tension than pure water. If they are allowed to escape drop by drop from a given volume of liquid through an aperture of constant dimension, the number of drops corresponding to each different mixture of alcohol and water is constant; and the number is, moreover, greater the higher the percentage of alcohol present. We have here then a means of estimating the percentage of alcohol in different aqueous solutions.

The instrument designed by Duclaux is a drop-counter with a capacity of 5 cc. between its upper mark and its lower extremity. This latter is formed by a capillary tube of such a diameter that the drops fall from it at the rate of about one per second. The apparatus is so constructed that when filled with distilled water at 15° it discharges exactly 100 drops (each instrument requires verification from this point of view).

499. When small quantities of alcohol in aqueous solution are to be measured, the pipette is filled to its upper mark by drawing up the liquid. It is then carefully dried, the contained liquid being kept in the pipette by closing the upper extremity with the index finger. It is then carefully placed in a vertical position in an appropriate bottle furnished with a cork with a suitable notch cut in the



margin; the finger is then removed and the number of drops falling into the bottle carefully counted (Fig. 54)

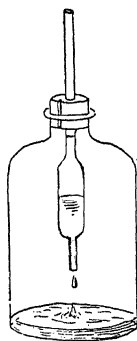


FIG. 54.—Duclaux's Drop-Counter.

At the same time the temperature must be read. If at the end of the operation a droplet remains at the end of the capillary tube it is counted as a half-drop.

Below are given the number of drops corresponding to different mixtures of alcohol and water at different temperatures :

TABLE SHOWING THE NUMBER OF DROPS CORRESPONDING TO DIFFERENT MIXTURES OF ALCOHOL AND WATER AT DIFFERENT TEMPERATURES, IN DUCLAUX'S APPARATUS (§ 499)

	12.5°.	15°.	17.5°.	20°.	22.5°.
Distilled water . . .	99.5	100	100.5	101	102
Alcohol .25 per cent .	101.5	102	102.5	103	104
" .5 " " "	103.5	104	104.5	105	106
" .75 " " "	105	105.5	106	106.5	107.5
" 1.0 " " "	106.5	107	107.5	108	109
" 2 " " "	112.5	113	113.5	114.5	115.5
" 3 " " "	117.5	118	118.5	119.5	120.5
" 4 " " "	122	122.5	123.5	124.5	125.5
" 5 " " "	125.5	126.5	127.5	128.5	130
" 6 " " "	129.5	130.5	131.5	132.5	134
" 7 " " "	133	134	135.5	136.5	138
" 8 " " "	136.5	137.5	139	140	141.5
" 9 " " "	139.5	140.5	142	143	144.5
" 10 " " "	142.5	144	145	146.5	147.5

500. *Chemical Method.*—A chemical method for estimating alcohol in very dilute solutions is based upon the following facts. If a mixture of 1 cc. of a 2 per cent solution of potassium bichromate, 5 cc. of a .1 per cent solution of alcohol, and 5 cc. of concentrated sulphuric acid is boiled for one minute and allowed to cool, the liquid appears pure green in colour. Now, if to a series of test-tubes we add varying quantities of .1 per cent alcohol solution, make the volume up to 5 cc. with distilled water, treat each of these tubes with the same quantities of potassium bichromate and sulphuric acid, boil for one minute and allow to cool, a series of tints will be obtained varying from green to yellow (a control tube being taken in which the 5 cc. of .1 per cent alcohol is replaced by water). These tubes can be kept for a long time if covered to avoid the entrance of dust and to retard evaporation. The quantities of .1 per cent alcohol suggested for this colour series are 5 cc., 4.5 cc., 4 cc., 3.5 cc., 3 cc., 2.5 cc., 1.5 cc., 1 cc., and .5 cc.

Solutions of alcohol under examination may be treated as above and their colour tints compared with the standards. The differences in colour in the above series are quite distinct, and with practice the values of intermediate stages can be estimated with sufficient accuracy.

501. The method of using the above colour series for the estimation of alcohol is as follows. A given quantity (say 5 cc.) of the liquid under examination is taken in a test-tube; 1 cc. of bichromate solution and 5 cc. of concentrated sulphuric acid are added; the mixture is boiled, allowed to cool, and its tint compared with the controls. If the colour is intermediate between green and yellow the amount of alcohol can be at once read off. If, however, it is pure green, the liquid contains *at least* .1 per cent alcohol, and a smaller quantity (made up to 5 cc. with distilled water) must be examined, until a tint comparable with that of one of the control tubes is obtained. The estimation can be made correctly to within  $\frac{1}{10}$ th or  $\frac{1}{20}$ th with a little practice.

### Estimation of Alcohol in Wine

502. To estimate the amount of alcohol present in a fermented liquid such as wine, beer, cider, etc., the alcohol is first separated by distillation.<sup>1</sup> To the distillate is added sufficient water to bring its volume up to that of the liquid distilled. A mixture of alcohol and water is thus obtained of the same alcoholic strength as the original liquid, and the percentage of alcohol may be estimated by taking its specific gravity with the Gay-Lussac's alcoholimeter. In using this method due care must be taken to eliminate any factors which might cause error in the reading of the specific gravity.

503. A known volume of wine (about 50 cc.) is taken to fill a Salleron's tube to its upper mark. The temperature of the wine, which should be 15° as nearly as possible, is taken, and it is put into a flask of 250 cc. capacity, rendered faintly alkaline to litmus with dilute soda solution, and distilled through a water-jacketed condenser. The distillate is collected in the vessel in which the wine was first measured, the delivery tube reaching to the bottom of the glass (so as to avoid loss by evaporation). In summer it is well to immerse the collecting vessel in ice-cold water.

When about two-thirds of the volume of the liquid originally taken have distilled over, one can be certain that all the alcohol originally present is now in the distillate. The distillation is stopped, and the volume of distillate made up to the mark on the measuring tube with distilled water. The added water must be either warm or cold, according to the temperatures of the distillate and that of the original wine. The final temperature of the mixture must of course in any case be the same as that of the wine when first measured. The water and distillate must be thoroughly mixed, and the thermometer adjusted in the groove of the

<sup>1</sup> In dealing with frothy liquids such as beer, they may be stirred in a vessel half-filled so as to get rid of as much CO<sub>2</sub> as possible. Another method is to pour the liquid repeatedly from one vessel into another. The distillation must be carried out very slowly, and a few drops of oil may be added to the contents of the distilling flask.

tube. The scale of the alcoholimeter should be carefully cleaned, either by wiping it with a fine linen rag or with Japanese paper moistened with ammonia so as to remove any greasy matter. After this it must not be touched again with the fingers, unless at its upper extremity, where there are no graduations. To take the reading the instrument should be gradually lowered in the liquid and left as soon as it is in equilibrium. If dropped too suddenly it falls and then rises again, so that a portion of the scale is

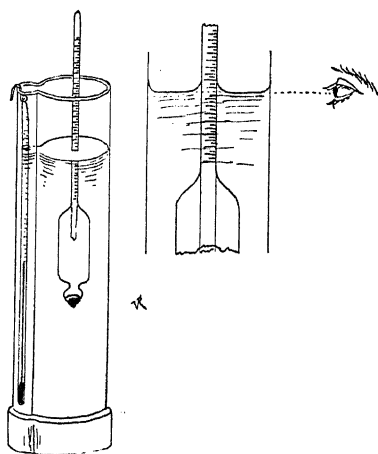


FIG. 55.—Alcoholimeter and method of reading.

moistened by the liquid and makes it too heavy; the readings so obtained would of course be too high.

When the instrument is at rest the reading is taken, but not at the summit of the meniscus. The eye must be placed on a level a very little below the surface, so as to see where the surface cuts the graduation on the scale (Fig. 55). At the same time the thermometer must be read and correction made according to the table. *The corrections must be added for temperatures below 15°, and subtracted for those above.*

When the only alcoholimeter available is one graduated from 0° to 10° the wine must be previously diluted with an

equal volume of water if its alcoholic reading is equal to or greater than 10°.

TEMPERATURE CORRECTIONS FOR ALCOHOLIMETER READINGS

Temperature.	Degrees measured on Alcoholimeter.													
	1°.	2°.	3°.	4°.	5°.	6°.	7°.	8°.	9°.	10°.	11°.	12°.	13°.	14°.
10	1'4	2'4	3'4	4'5	5'5	6'5	7'5	8'5	9'5	10'6	11'7	12'7	13'8	14'9
11	1'3	2'4	3'4	4'4	5'4	6'4	7'4	8'4	9'4	10'5	11'6	12'6	13'6	14'7
12	1'2	2'3	3'3	4'3	5'3	6'3	7'3	8'3	9'3	10'4	11'5	12'5	13'5	14'6
13	1'2	2'2	3'2	4'2	5'2	6'2	7'2	8'2	9'2	10'3	11'4	12'4	13'4	14'4
14	1'1	2'1	3'1	4'1	5'1	6'1	7'1	8'1	9'1	10'2	11'2	12'2	13'2	14'2
16	0'9	1'9	2'9	3'9	4'9	5'9	6'9	7'9	8'9	9'9	10'9	11'9	12'9	13'9
17	0'8	1'8	2'8	3'8	4'8	5'8	6'8	7'8	8'8	9'8	10'8	11'7	12'7	13'7
18	0'7	1'7	2'7	3'7	4'7	5'7	6'7	7'7	8'7	9'7	10'7	11'6	12'5	13'5
19	0'6	1'6	2'6	3'6	4'6	5'5	6'5	7'5	8'5	9'5	10'5	11'4	12'4	13'3
20	0'5	1'5	2'4	3'4	4'4	5'4	6'4	7'3	8'3	9'3	10'3	11'2	12'2	13'1
21	0'4	1'4	2'3	3'3	4'3	5'2	6'2	7'1	8'1	9'1	10'1	11'0	11'9	12'8
22	0'3	1'3	2'2	3'2	4'1	5'1	6'1	7'0	7'9	8'9	9'9	10'8	11'7	12'6

### Detection of Aldehyde

504. Ordinary (acetic) aldehyde is produced in various fermentations, either by direct action of micro-organisms or by oxidation of alcohol through contact with the air.

To show the presence of aldehyde the liquid is distilled, having been neutralized if its reaction is alkaline. The distillate is tested with ammoniacal silver nitrate solution or with Nessler's reagent, both of which are rapidly reduced by aldehyde. A further test is with concentrated nitric acid containing .5 per cent potassium bichromate, which gives a violet-blue in the cold in the presence of aldehyde. Treated as described in § 495 the distillate gives the iodoform reaction.

### Estimation of Aldehyde

505. The amount of aldehyde present in a given solution can be estimated by using its capability of combining with alkaline bisulphites. An excess of bisulphite is used for

the estimation, the amount of sulphurous acid before and after the action of aldehyde being titrated by means of a decinormal solution of iodine, whence the amount of aldehyde originally present can be calculated.

After distillation 100 cc. of the distillate are measured in a graduated flask; to this is added a known quantity (but in excess) of decinormal potassium bisulphite solution;<sup>1</sup> for instance, 20 cc. The mixture is shaken in a well-stoppered flask of 250 cc. capacity and left for a quarter of an hour with occasional shaking. Decinormal iodine is then run in, until a faint yellow just persists after shaking, and the volume of iodine solution employed is read off.

The operation is repeated, using 100 cc. of water and 20 cc. of bisulphite solution, and decinormal iodine run in as before. The difference between the two readings gives the quantity corresponding to the aldehyde. From the equation  $\text{KHSO}_3 + \text{I}_2 + \text{H}_2\text{O} = \text{KHSO}_4 + 2\text{HI}$ , it is seen that each cubic centimetre of decinormal iodine corresponds to .5 cc. of decinormal bisulphite or to .5 cc. of decinormal aldehyde, and so to .0022 gramme of aldehyde itself.

### Detection of Acetone

506. Acetone is produced in a number of microbial fermentations. If present in small amount, the liquid suspected to contain it is distilled in an Aubin apparatus so as to concentrate the acetone in the first portions of the distillate. If needful this distillation is repeated. The final distillate is used for the following tests.

507. Ten cubic centimetres of the distillate are taken in a test-tube, one drop of a 10 per cent solution of hydroxylamine hydrochloride, one drop of 5 per cent soda, two drops of pyridine, and 1 or 2 cc. of ether are added. The mixture is then oxidised by bromine water, added drop by drop, until the ether attains a yellow colour. The colour changes to pale blue, either by simple shaking with air or by the addition of a drop of hydrogen peroxide.

<sup>1</sup> A solution of crystallized potassium metabisulphite  $\text{K}_2\text{S}_2\text{O}_5$ , 6 grammes per litre.

508. The presence of acetone can also be shown by the formation of *p*-nitrophenylhydrazone. To 1 cc. of the distillate are added 5 cc. of 96 per cent alcohol, and the mixture is saturated with *p*-nitrophenylhydrazine at the ordinary temperature. To the liquid so obtained are added five times its volume of distilled water; the resulting yellow crystalline precipitate is collected and dried. It is purified by re-solution in alcohol and again precipitated by the addition of five volumes of water. The crystals melt at 148° (using Maquenne's block); treated with an alcoholic solution of potash they turn orange-red.

509. Even very dilute solutions of acetone when treated with Denigès' reagent (§ 180) throw down a dense white precipitate of the formula  $(2\text{HgSO}_4, 3\text{HgO})_3 + 4\text{CH}_3 \cdot \text{CO} \cdot \text{CH}_3$ . This reaction is given by all bodies containing a ketone group.

510. The distillate when treated as in § 495 gives the iodoform reaction similarly to alcohol and aldehyde. From these it is distinguished by the fact that it does not modify the yellow tint of a .5 per cent solution of potassium bichromate in concentrated nitric acid.

511. *Detection of Acetone in Urine.*—Certain pathological urines contain acetone in varying amounts. Its presence can be shown by the following method:

To 5 cc. of urine in a test-tube is added a small crystal of sodium nitroprusside (about twice the size of a pin's head). The mixture is shaken to promote solution, and two or three drops of soda solution are added. A red coloration is produced if acetone is present (Legal's test); the reaction is, however, also given by creatinine. If now sufficient acetic acid is added to render the liquid distinctly acid, the colour changes to reddish violet if acetone is present. If there is no acetone present the solution is decolorized.

### Estimation of Acetone

512. The quantitative determination of acetone is based upon the formation of iodoform in the presence of a known

excess of iodine. The free iodine remaining is titrated when the combination is complete, and hence the amount fixed by the acetone can be determined.

In the first place, 100 cc. of the liquid under examination are rendered slightly acid by the addition of .5 cc. of acetic acid and then distilled. The distillate is collected in a wide-mouthed bottle of about 500 cc. (which can be fitted with a well-ground glass stopper) and kept cool in ice-cold water. When about 80 cc. of distillate have been collected distillation is stopped and 30 cc. of soda solution are added. Decinormal iodine is next run in until the iodine is just present in excess. One can recognize when this point is reached by allowing a drop of hydrochloric acid to fall into the flask (the contents being kept in gentle movement), when it produces a brown coloration.

The volume of iodine used is carefully noted. After five or six minutes, during which the bottle is frequently shaken, the reaction is finished and the iodoform falls to the bottom of the bottle. The mixture is now rendered acid by the addition of 50 per cent hydrochloric acid (using a piece of litmus-paper as indicator). The amount of free iodine remaining is titrated by means of a decinormal solution of sodium thiosulphate, which is run in until the yellow colour just disappears. The volume necessary for this, deducted from the total volume of iodine solution used, gives the quantity of iodine necessary for the formation of iodoform. Each cubic centimetre of iodine (decinormal) solution corresponds to .967 mgr. of acetone.

### Detection of Acetylmethylcarbinol

513. Acetylmethylcarbinol,  $\text{CH}_3 \cdot \text{CO} \cdot \text{CHOH} \cdot \text{CH}_3$ , is produced in small amounts in several fermentations.<sup>1</sup> For its detection the culture fluid is first neutralized, then distilled. A sufficient amount of alkali-copper solution is added

<sup>1</sup> Acetylmethylcarbinol is often accompanied by small amounts of 2,3. butyleneglycol,  $\text{CH}_3 \cdot \text{CHOH} \cdot \text{CHOH} \cdot \text{CH}_3$ , which, however, does not reduce alkali-copper solutions.



to a few cubic centimetres of the distillate to colour it a bright blue. The mixture is left *at the ordinary temperature* for a few seconds, when in the presence of acetylmethylcarbinol reduction occurs.

514. To make sure that this reduction is due to the presence of acetylmethylcarbinol, to a portion of the distillate is added  $\frac{1}{10}$ th of its volume of phenylhydrazine acetate (see § 91), and the mixture is heated on the boiling water-bath for thirty to forty minutes. An osazone crystallizing in bright yellow needles is formed. On cooling, these can be filtered off and washed with cold water.

515. If a small quantity of these crystals is placed in a test-tube with 5 cc. of alcohol and an equal volume of ether, the cautious addition of ferric chloride solution gives a deep red colour. On adding an equal volume of water to the mixture the ether separates out and is coloured red. It is decanted off and evaporated in a small capsule. A deposit of long dark red needles is left; these are insoluble in water and melt at  $151^{\circ}$ .

516. The following test is also used. Mix in a test-tube 3 cc. of a 1 per cent Witte peptone solution with an equal volume of 10 per cent soda solution, and carefully run on to the surface of the mixture 2 or 3 cc. of the distillate. At the line of separation of the two liquids a red ring appears (Voges and Proskauer).

### Detection and Separation of Lactic Acid

517. Lactic acid is produced in varying amounts in a large number of fermentations.

To extract it from a culture-liquid this is heated on the water-bath, and oxalic acid is added (in as exact a quantity as possible) to precipitate calcium salts. The liquid is filtered off and the precipitate washed on the filter, the washings being added to the filtrate, which is then evaporated to a syrupy consistency. This strongly acid syrup is extracted by half a dozen shakings with ether previously saturated with water. Four or five volumes of

ether must be used to one volume of syrup for each extraction. The ethereal extracts are mixed, filtered, and then distilled. Lactic acid, if present, remains in the syrupy residue in the distilling-flask; it can be recognized by the usual reactions (§ 170 *et seq.*).

518. To isolate lactic acid as its zinc salt, the residue mentioned above is dissolved in water and baryta is added in slight excess. After allowing it to stand some time (necessary for the transformation of any lactyl-lactic ester present, § 185), the excess of baryta is treated with carbon-dioxide. The mixture is boiled to decompose the barium bicarbonate, filtered, and the filtrate exactly precipitated with 10 per cent zinc sulphate solution. Finally the barium sulphate is filtered off and the filtrate concentrated by evaporation. On cooling, zinc lactate crystallizes out. After twenty-four hours it is collected and purified by recrystallization from water. For its properties see § 173.

519. When the culture-liquid under examination contains a large amount of lactic acid, as in the typical lactic fermentation (§ 477), its presence can be shown without preliminary extraction with ether. For this purpose the liquid is filtered as soon as the fermentation is finished (generally after five or six days), brought to boiling point, and a saturated solution of oxalic acid added, so as to precipitate all the calcium present. A very small excess of oxalic acid may be present. The mixture is filtered and the clear filtrate is boiled for half an hour with excess of zinc carbonate. This is again filtered to separate the insoluble carbonate and oxalate of zinc; the filtrate is evaporated on the water-bath, and on cooling deposits a crop of crystals of zinc lactate. Zinc lactate, microscopically, appears as long prismatic crystals often grouped in rosettes (Fig. 22).

### Detection and Estimation of Succinic Acid

520. For these purposes the method described in § 184 (which is of general application) is employed.

### Identification and Estimation of Volatile Acids (Duclaux's Method)

521. This method is specially applicable to the estimation of volatile acids in fermented liquids. The first step necessary is the removal of volatile bodies which are not acids (such as alcohols), by neutralizing and then distilling the mixture till only a third of its original volume remains. The volatile acids are then obtained in a free state by the addition of a non-volatile acid in slight excess. Tartaric acid is useful for this purpose, because on allowing the mixture to stand for a few hours any calcium salts present (and this is often the case) yield a crystalline precipitate of calcium tartrate which can easily be separated by decantation. In so freeing the liquid from the whole or part of its contained calcium the amount of non-volatile substances present is reduced, which favours the accuracy of the determination of volatile acids. This determination is always most satisfactory when the solution examined approximates closely to a simple aqueous solution of volatile acids.

522. *Principle of the Method.*—This is as follows. Suppose a solution of some volatile acid taken of about 1 per cent, or at the maximum 2 per cent. This is made up to a definite volume, for example, 110 cc., and distilled in a flask of 250-300 cc. capacity fitted with an ordinary condenser. The distillate is collected in ten successive quantities each of exactly 10 cc. Each of these fractions is titrated as regards its acidity, and the different readings carefully noted. Suppose that the total quantity of acid introduced into the flask required 100 cc. of the alkaline solution, the successive readings represent the proportions (in hundredths) of acid in the first 10, 20, 30, etc., cubic centimetres distilled over. This granted, the three following laws may be assumed :

- (1) The sequence of numbers occurring in these titrations is characteristic of the volatile acid used.
- (2) A constant relation exists between the amount of

acid in the distilling flask and the quantity distilling over at any given time, so that from the quantity distilled in the first 10, 20, 30, etc., cubic centimetres, the total amount of acid originally introduced into the distilling flask can be judged.

- (3) If there is a mixture of two acids, each behaves as if it were present alone and follows its own laws of distillation.

The practical application of these laws may now be considered.

523. *Case of a Single Acid.*—First let us consider the case when only a single acid is present in the liquid to be distilled. Exactly 110 cc. are placed in a flask of about 250 cc. capacity, and distilled at such a rate that the duration of the operation does not exceed forty or forty-five minutes. The distillate is collected in little graduated flasks, each to contain 10 cc. The end of the condenser tube should be bevelled and adjusted at such a height that it just rests lightly upon the neck of the collecting-flask. The progress of the distillation is carefully watched, and as soon as the distillate reaches the mark on the flask, the latter is quickly but carefully removed and replaced by a second flask. Should the first flask contain an excess this must be removed from the surface with a finely-drawn-out pipette and transferred to the second flask.

The first 10 cc. fraction of distillate is immediately turned into a large glass capable of holding 250-300 cc. The flask is washed out three times with successive quantities of 2-3 cc. of distilled water, the washings being added to the contents of the glass. The flask is then carefully drained by inverting it upon some layers of filter-paper. The first fraction of distillate is then neutralized by an alkali. Lime-water is convenient for this purpose, since when saturated it is of a very suitable degree of concentration for such estimations. The lime-water is run in from a burette, and phenolphthalein is used as indicator. When the second collecting-flask is filled to the 10 cc. mark it is replaced by the first flask. The contents of the second

flask are added to the contents of the large glass, and again neutralized with lime-water, but the burette is not re-adjusted to zero. The same procedure is adopted for the remaining eight fractions, when the distillation is stopped to avoid breakage of the distilling-flask.

If now we examine the relations between the volumes of lime-water necessary to saturate the first 10, 20, 30, etc., cubic centimetres of distillate—compared with the volume, 100 cubic centimetres, required to neutralize the whole volatile acid content of the distilling-flask—the numbers obtained for the volatile acids usually found in fermentation processes will be found to be as follows :

FIRST TABLE

	Formic Acid.	Acetic Acid.	Propionic Acid.	Butyric Acid.	Valerianic Acid.
10 cc.	3'5	5'9	11'5	17'3	30'5
20 cc.	7'2	12'2	22'8	32'7	53'0
30 cc.	11'3	18'7	33'5	47'0	69'5
40 cc.	15'5	25'6	44'0	58'5	81'0
50 cc.	20'2	32'7	54'0	68'8	88'5
60 cc.	25'5	40'4	63'3	77'5	93'5
70 cc.	31'1	48'7	72'5	84'3	96'5
80 cc.	38'5	57'5	81'0	90'5	98'3
90 cc.	48'0	67'5	88'5	94'6	99'5
100 cc.	59'0	80'0	95'0	97'5	100'0

The relations between these various distillations are shown in the accompanying series of curves (Fig. 56). It will be noticed that it is the less volatile acids which pass over most readily in the first stages of the distillation. With formic and acetic acids the titre of the different fractions increases constantly. With propionic acid there is a progressive lessening of the titre, but it is slow. This lessening is however rapid in the case of butyric acid, and still more so in that of valerianic acid, both of which tend to concentrate in the distillate, while formic and acetic acids tend to concentrate in the liquid contained in the

distilling-flask, as may be seen from the figures given in the table.

It follows from this that a reading of the figures obtained in successive titrations will enable us to determine what acid is present; and then, by considering any particular fraction, a reference to the above table will give the factor which enables us to determine the quantity of acid present

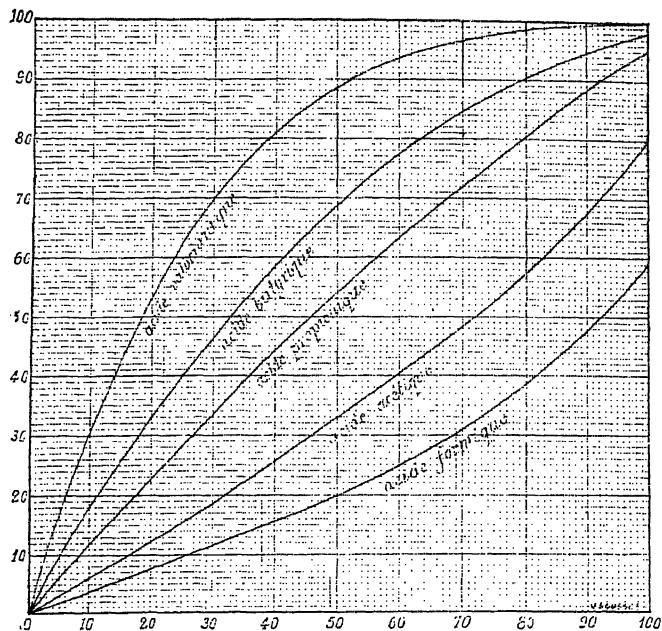


FIG. 56.

in the distilling-flask. For example, if ten fractions (*i.e.* 100 cc.) have been collected, and the series of figures obtained corresponds to that for acetic acid, the total amount of acid present in the distilling-flask will be found by multiplying the quantity of acid found in the ten fractions by the factor corresponding to that for 100 cc. in the acetic acid column in the first table; as this is 80, the total amount of acid originally present will be given by multiplying

the amount of acid found in 100 cc. by the factor  $100/80$  or  $5/4$ .

The *name* then of the acid with which we are dealing will be ascertained by consideration of the sequence of figures obtained in examining successive fractions of distillate. Instead of computing these figures as percentages of the acid in the flask (of which nothing is initially known), it is more convenient to calculate them as percentages of the total acid contained in 100 cc. of distillate. These figures will be obtained by taking the ratios of the various numbers in each column to the figure at the foot of the column. The following numbers are then obtained :

SECOND TABLE

	Formic Acid.	Acetic Acid.	Propionic Acid.	Butyric Acid.	Valerianic Acid.
10 cc.	5.9	7.4	12.1	17.6	30.5
20 cc.	12.2	15.2	24.0	33.6	53.0
30 cc.	19.0	23.4	35.3	47.5	69.5
40 cc.	26.4	32.0	46.2	60.0	81.0
50 cc.	34.4	40.9	56.8	70.6	88.5
60 cc.	43.2	50.5	66.7	79.5	93.5
70 cc.	52.8	60.9	76.2	86.5	96.5
80 cc.	64.6	71.9	85.0	92.5	98.3
90 cc.	79.6	84.4	93.0	97.0	99.5
100 cc.	100.0	100.0	100.0	100.0	100.0

The subjoined curves (Fig. 57) again show the sequence of the values so obtained. It will be noticed that they are less separated than the preceding set of curves (Fig. 56). Commencing to separate from a common point zero, they meet again at 100. They are, however, sufficiently distinct from one another to be characteristic of the different acids they represent. It is possible, therefore, by comparing the percentage readings obtained in any given experiment, with the figures in the second table, to ascertain the acid with which we are dealing ; and then, from the quantity of acid which has distilled over, to calculate, by means of

the first table, the *total quantity* originally present in the sample submitted to distillation.

524. *Examination of a Mixture of Two Acids.*—When two volatile acids are present the investigation is a little more complicated. It has already been noted that under such circumstances each acid behaves as if it alone were present and follows the typical course of its distillation.

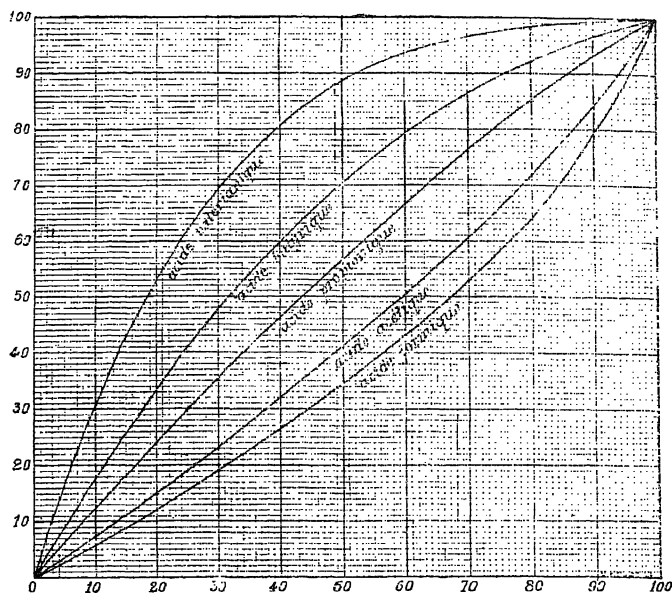


FIG. 57.

If, for example, we are dealing with a mixture of equivalent parts of acetic and butyric acids, the sequence of figures obtained by titration of the different fractions of distillate will be the mean of the numbers corresponding to each of the two acids in either of the preceding tables, and the distillation curve will be that which in either of the preceding diagrams is vertically equidistant from those of acetic and butyric acids.



If, on the contrary, there are two molecules of butyric acid to one of acetic, it will be necessary to add double the numbers corresponding to butyric acid to those of acetic acid, and to take one-third of their sum.

Conversely, by plotting the actual figures obtained in a given experiment it can be seen whether the curve corresponds with one of those given in Fig. 57, and if this is not the case, between which two curves the experimental curve lies. When this curve is once determined the question of the identification of the two acids under investigation resolves itself into a methodical series of tentative observations which can be interpreted with a little experience.

With a mixture of formic and acetic acids, where both curves have their convexities directed towards the same side the distillation curve will have the same form, and the numbers will show progressive increase from the first to the last.

With mixtures of butyric and valerianic acids, of which the curves also have their convexities in the same direction (but opposite to those of formic and acetic acids), the distillation curve will be intermediate in position between those of the two acids concerned: the numbers obtained from successive fractions being found to show progressive diminution and values intermediate between those obtaining for these two acids separately. Let us now consider the case of two acids which present distillation curves with their convexities facing opposite directions, and, taking two extreme instances, consider a mixture of equivalent parts of formic and valerianic acids. The numbers corresponding to the distillation of this mixture are the following, which are obtained by taking the mean of the figures corresponding to these two acids in the second table:

10	20	30	40	50	60	70	80	90	100
18.2	32.6	44.2	53.7	61.5	68.4	74.6	81.5	89.5	100

The corresponding curve (1 in Fig. 58) is a double curve where the titre of the successive fractions diminishes at the

beginning, because the valerianic acid distils over at this time, and then increases owing to the large amount of formic acid passing over.

Similarly a mixture of two equivalents of valerianic acid with one of formic acid gives curve 2 (Fig. 58); while a mixture of two equivalents of formic with one of valerianic gives curve 3 (Fig. 58). From these it is seen that the

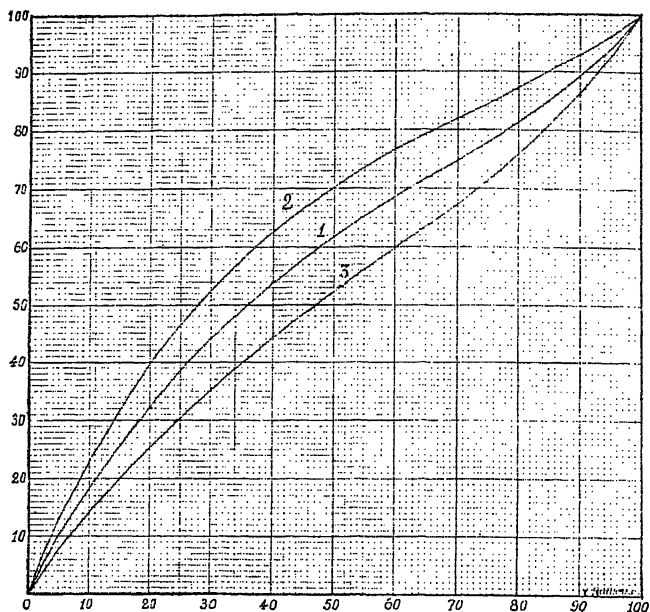


FIG. 58.

point of inflexion of the curve varies with the proportions of the mixed acids.

A curve with a double flexure, however small it may be, or an irregularity in the increase or decrease of the figures obtained in the successive titrations, denotes the presence of a mixture of an acid with a greater number of carbon atoms than propionic acid, with either formic or acetic acid. The precise character of the mixture can be determined

either by plotting the figures obtained by titration of the distillates, or by comparison with the figures given in the tables (pp. 304-306).

525. *Nature of the Acids present in Mixtures.*—Here the problem is a double one: firstly to determine what acids are present, and secondly their relative proportions.

In the various investigations into the nature of the acids present one employs every available means. Thus valerianic is easily distinguished from butyric acid by its odour; formic acid differs from acetic acid in reducing ammoniacal silver nitrate solution. Generally speaking, when the form of the curve indicates a mixture of formic or acetic with butyric or valerianic acid, it is easy to determine with which pair of acids we have to deal. The case of propionic acid is considered in a subsequent paragraph.

To sum up. The regular sequence of the values obtained by titrating successive fractions of distillate, or the regular forms of the curves, informs us that we are dealing with mixtures of acetic and formic acids, or of butyric and valerianic acids. On the other hand, an irregular sequence of figures, first showing a diminution and then an increase for the numbers obtained with successive fractions of distillate, or a double curve, denotes a mixture of acetic or formic acid with butyric or valerianic acid. Generally speaking, it is easy—unless one of the acids should only be present in feeble quantity—to give a name to the mixture of the two acids in question; and then it only remains to estimate their relative proportions.

526. *Proportions of Acids present in Mixtures.*—Here again the required information will be gathered from a consideration either of the various figures or of the curves. The experimental figures, in so far as intermediate between the normal figures for any pair of acids, divide the difference between the normal figures into parts which are inversely proportional to the quantities of each acid present in the liquid examined. If, then, we take the difference between any experimental number and the

corresponding higher and lower numbers of Table 2, the ratio of these findings will give an approximate estimate of the proportion of the lower to the higher acid. In this way, nine such approximate values may be estimated, and their mean value determined; but it is evident that the numbers corresponding to where the curves are the most widely divergent will give more accurate findings than those relating to the commencement and the end of the experiment, where the curves tend to approximate very closely. In practice the mean of the proportions corresponding to the 30, 40, 50, 60, 70 cc. fractions only is considered, the desired ratio being easily calculated therefrom. The degree of accuracy thus attained is greater than that afforded by any other analytical procedure at present known.

Once this ratio is known no further difficulty presents itself. By means of the first table we find what mixture of acids should distil over at such a rate as to give the ratio found, and from the volume of lime-water used to saturate the actual distillate to determine that which would be necessary for the total volatile acids contained in the flask. Knowing the amount of lime-water necessary to neutralize the acids in the mixture, the titre of the lime-water, and the equivalent proportions of the mixed acids, the actual quantities of each acid present in the mixture can be determined.

527. *Propionic Acid*.—We now return to the consideration of propionic acid, which is intermediate in position between acetic and butyric acids. The sequence of figures obtained for the titration of successive fractions of this acid is almost identical with the mean of those of equivalent quantities of the two neighbouring acids—acetic and butyric.

The differences are better seen by comparing the curves, because propionic acid preserves the regular form of curve definitely, although not to a marked degree, whereas the curve of mixtures is a double curve such as previously described. Nevertheless, since the differences are small,

there is danger of confusing a curve due to propionic acid with that due to a mixture of butyric and acetic acids. Fortunately we have a simple means of evading this difficulty, and that is to distil the liquid under examination until one half has passed over. This distillate is to be collected in two equal fractions. If we are dealing with a simple solution of a single acid these two fractions will contain the same acid, and on being subjected to a further fractional distillation (10 or 11 fractions) the same figures will be obtained on titration.

If, on the contrary, it is a case of a mixture of butyric and acetic acids, butyric acid will tend to concentrate in the first half, while acetic acid will tend to concentrate in the second half. On fractionally redistilling, the fractions of the two halves will give such different results that all doubt will be set at rest.

This process of fractioning into two, three, or four parts, according to circumstances, is always to be recommended with mixtures, where one acid is present in very small amount relatively to the other, when distillation *en bloc* would leave the interpretation of the results open to doubt. It is then always possible by distillation to concentrate butyric or valerianic acids in the first portions and acetic or formic acids in the last, so that they are present in sufficient quantity to allow of their recognition.

Finally, this method of preliminary distillation is useful with mixtures of three acids. Such mixtures are, however, very rare as the result of a single pure fermentation, and when they do occur one of the acids—generally that with the highest molecular weight—is only present in infinitesimal amount. For the separation advantage is taken of the fact that in the first twenty cubic centimetres of the distillate is contained half the valerianic acid, and sixty per cent of the caproic. The residue in the distilling-flask is that freed from a large proportion of the third acid, and what remains is present in too small an amount to interfere with the normal progress of the determination.

The experiments and calculations relating to the different proportions of acid mixtures can be facilitated by reference to the following tables, which give the sequences of figures obtained with the most common mixtures of two acids commonly met with in fermentation processes. In all these mixtures it is only necessary to tabulate the ratios for the region of widest deviation between the curves where the measurements admit of the greatest precision. This region varies with different mixtures, and is indicated by the volumes of the fractions at which it commences and terminates.

Subjoined are the figures for different mixtures of acids, the proportions of each constituent being indicated in the first column: the relations given can be calculated from the figures given in the second table (p. 297).

MIXTURES OF VALERIANIC AND ACETIC ACIDS

	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.
Pure valerianic acid . .	53·0	69·5	81·0	88·5	93·5	96·5
20 ac. valer. : 1 ac. acet. .	52·2	67·3	78·6	86·2	91·4	94·8
10   "    1   "    .	49·5	65·3	76·5	84·2	89·6	93·3
8   "    1   "    .	48·8	64·4	74·4	83·2	88·7	92·5
6   "    1   "    .	47·6	62·9	74·0	81·7	87·3	91·4
5   "    1   "    .	46·7	61·8	72·8	80·6	86·1	90·6
4   "    1   "    .	45·4	60·2	71·2	79·0	84·9	89·4
3   "    1   "    .	43·5	58·0	68·8	76·6	82·8	87·6
2   "    1   "    .	40·4	54·1	64·7	70·1	79·2	84·6
1   "    1   "    .	34·1	46·4	56·5	64·7	77·0	78·7
1   "    2   "    .	27·8	38·8	48·3	56·8	64·8	72·8
1   "    3   "    .	24·6	34·4	44·4	52·8	61·2	69·8
1   "    4   "    .	22·8	32·6	41·8	50·4	59·1	67·8
1   "    5   "    .	21·5	31·1	40·1	48·8	57·7	66·8
1   "    6   "    .	20·6	30·0	39·0	47·7	56·6	66·0
1   "    8   "    .	19·4	28·5	37·4	46·2	55·3	64·8
1   "    10   "    .	18·6	27·6	36·4	45·2	54·4	64·1
1   "    20   "    .	17·0	25·1	34·3	43·1	52·5	63·1
Pure acetic acid . .	15·2	23·4	32·0	40·9	50·5	60·9

## MIXTURES OF BUTYRIC AND ACETIC ACIDS

	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	
Pure butyric acid . . .	47.5	60.0	70.6	79.5	86.5	92.5
10 ac. butyr. : 1 ac. acet. .	45.3	57.5	67.9	76.9	84.2	90.6
5 " " 1 " " .	43.5	55.3	65.6	74.7	82.2	89.1
4 " " 1 " " .	42.6	54.4	64.6	73.7	83.0	88.4
3 " " 1 " " .	41.5	53.0	63.2	72.2	80.1	87.3
2 " " 1 " " .	39.5	50.8	60.7	69.8	78.0	85.6
1 " " 1 " " .	35.2	46.0	55.7	65.0	73.7	82.2
1 " " 2 " " .	31.4	41.3	50.8	60.2	69.4	78.8
1 " " 3 " " .	29.4	39.0	48.8	57.7	67.3	77.0
1 " " 4 " " .	28.2	37.6	46.8	56.3	66.0	76.0
1 " " 5 " " .	27.4	36.7	45.8	55.3	65.2	75.3
1 " " 10 " " .	25.6	34.5	43.6	53.1	63.2	73.8

## MIXTURES OF PROPIONIC AND ACETIC ACIDS

	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.
Pure propionic acid . . .	35.3	46.2	56.8	66.7	76.2	85.0
5 ac. propion. : 1 ac. acet. .	33.3	43.8	54.2	64.0	73.6	82.8
4 " " 1 " " .	32.9	43.3	53.6	63.4	73.0	82.2
3 " " 1 " " .	32.3	42.6	52.8	62.6	72.4	81.7
2 " " 1 " " .	31.3	41.1	51.9	61.3	71.1	80.6
1 " " 1 " " .	29.3	39.1	48.9	58.6	68.5	78.5
1 " " 2 " " .	27.4	36.7	46.2	55.9	66.0	76.3
1 " " 3 " " .	26.4	35.5	44.9	54.5	64.7	75.2
1 " " 4 " " .	25.8	34.8	44.1	53.7	64.0	74.5
1 " " 5 " " .	25.4	34.4	43.6	53.2	63.5	74.0

## MIXTURES OF VALERIANIC AND BUTYRIC ACIDS

	10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.
Pure valerianic acid . . .	30.5	53.0	69.5	81.0	88.5	93.5
10 ac. valer. : 1 ac. butyr. .	29.3	51.2	67.5	79.1	86.9	92.2
5 " " 1 " " .	28.3	40.8	65.8	77.5	85.5	91.2
4 " " 1 " " .	27.9	49.1	65.1	76.8	84.9	90.7
3 " " 1 " " .	27.3	48.1	64.0	75.8	84.0	90.0
2 " " 1 " " .	26.2	46.5	62.2	74.0	82.5	88.8
1 " " 1 " " .	24.1	43.3	58.5	70.5	79.5	86.5
1 " " 2 " " .	21.9	40.1	54.8	67.0	76.6	84.2
1 " " 3 " " .	20.8	38.4	52.8	65.2	75.1	83.0
1 " " 4 " " .	20.2	37.5	51.9	64.2	74.2	82.2
1 " " 5 " " .	19.8	37.0	51.2	63.5	73.6	81.8
1 " " 10 " " .	18.8	35.4	49.5	61.8	72.2	80.8
Pure butyric acid . . .	17.6	33.6	47.5	60.0	70.6	79.5

## MIXTURES OF VALERIANIC AND PROPIONIC ACIDS

	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.
Pure valerianic acid . . . . .	53·0	69·5	81·0	88·5	93·5	96·5
10 ac. valer. : 1 ac. propion.	50·4	66·4	77·8	85·5	91·0	94·6
5   "    I    "	48·2	63·8	75·2	83·2	89·0	93·2
4   "    I    "	47·2	62·6	74·0	82·2	88·1	92·4
3   "    I    "	45·7	60·9	72·3	80·6	86·8	91·4
2   "    I    "	43·3	58·1	69·4	77·9	84·6	89·7
I   "    I    "	38·5	52·4	63·6	77·6	80·1	86·3
I   "    2    "	33·7	46·7	57·8	66·7	75·6	83·0
I   "    3    "	31·2	43·8	54·8	64·7	73·4	81·3
I   "    4    "	29·8	42·1	53·2	63·2	72·1	80·2
I   "    5    "	28·8	41·0	52·0	62·1	71·0	79·6
I   "    10   "	26·6	38·4	48·4	59·7	69·1	78·0
Pure propionic acid . . . . .	24·0	35·3	46·2	56·8	66·7	76·2

## MIXTURES OF BUTYRIC AND PROPIONIC ACIDS

	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.
Pure butyric acid . . . . .	33·6	47·5	60·0	70·6	79·5	86·5
8 cc. ac. butyr. : 1 ac. propion	32·5	46·1	58·5	69·1	78·1	85·3
4   "    I    "	31·7	45·0	57·2	67·8	77·0	84·4
3   "    I    "	31·2	44·4	56·5	67·1	76·3	83·9
2   "    I    "	30·4	43·4	55·4	66·0	75·1	83·3
I   "    I    "	28·8	41·4	53·1	63·7	73·1	81·3
I   "    2    "	27·2	39·4	50·8	61·2	71·0	79·6
I   "    3    "	26·6	38·3	49·6	60·2	69·9	78·8
I   "    4    "	25·9	37·7	49·0	59·5	69·2	78·2
I   "    8    "	25·0	36·7	47·7	58·3	68·1	77·3
Pure propionic acid. . . . .	24·0	35·3	46·2	56·8	66·7	76·2

These tables will be found of great assistance in making the necessary calculations. When once the set of percentage ratios for any given fermentation is known, a little practice enables us to ascertain from the sequence of readings, and by the odours of the earliest and last fractions, with what acids we have to deal, and these indications are checked by comparing the experimental figures with those given in the tables, and observing with which they are in closest accord. Complete agreement between the figures



in any experiment and those given in the tables is of course not to be expected; irregularities in the course of distillation, small errors in collecting the various fractions, and experimental error in the titrations themselves all tend to produce minor discrepancies. One has to be content with theoretical and experimental figures in agreement to 1 or 2 units in the first place of decimals. When the experimental series agrees with a series in the tables within these limits, it is sufficient to establish the qualitative and quantitative composition of the mixture with which we are dealing. It will often happen that the series of figures obtained in an experiment will fall between two consecutive series in the table; sometimes midway between them, and sometimes inclining to the one or the other. A simple mental calculation suffices to give an idea of the proportions of the two acids in the mixture.

### Detection of Ammonia

528. Some nitrogen-containing bodies produce ammonia as the result of bacterial action; the ammonia then exists as ammonium salts in the culture media. Frequently ammonia can be recognized directly in the medium itself by means of Nessler's reagent.<sup>1</sup> For this purpose 1 cc. of the reagent should be added to 10 cc. of the liquid examined. When large quantities of ammonia are present a reddish-brown precipitate is formed; in the presence of traces of ammonia only a brownish-yellow colour will be observed. The reaction is perfectly definite with 1 part of ammonia in 500,000.

529. If 10 cc. of a solution containing ammonia and 1 cc. of 10 per cent potassium iodide are mixed in a test-tube, and

<sup>1</sup> To prepare this reagent, 20 grammes of KI are dissolved in 50 cc. of water and heated on the water-bath, then with constant stirring red iodide of mercury as long as it dissolves (40-50 grammes); 200 cc. of water are next added, the mixture is allowed to cool, and then filtered. The solution of potassio-mercuric iodide then has an equal volume of 20 per cent soda solution added; the mixture is allowed to stand for twenty-four hours, when the clear liquid is decanted.

a few drops of an alkaline hypochlorite (commercial "Eau-de-Javel" for instance) are added, a black precipitate results. This reaction, though somewhat evanescent, is very delicate (Trillat and Turchet). Similarly to the reaction with Nessler's reagent, however, it is also given by a certain number of amines.

530. Upon the addition of sodium hypobromite, ammonia and ammonium salts evolve gaseous nitrogen. This reaction is also given by urea (§ 327), uric and hippuric acids, creatinine, etc. It is not given by amines.

531. To 10 cc. of the neutral or alkaline liquid are added 2 cc. of a 4 per cent watery solution of phenol, and 2-5 drops of freshly prepared eau-de-Javel (excess of this must be avoided); in the presence of ammonia a blue colour results. This reaction is as sensitive as that with Nessler's reagent. It is also given by glycocoll and some other bodies containing amino groups, but only when these are in relatively high concentrations (P. Thomas).

532. In order to eliminate any possibility of error it is far more satisfactory to distil the culture fluid with a little freshly calcined magnesia. The ammoniacal distillate is collected in a tube containing 4-5 drops of 10 per cent sulphuric acid. The foregoing tests can then be applied to the distillate.

### Estimation of Ammonia

533. *Titration of Ammonia formed in Fermentation Processes.*—The procedure is the same both for ammonia produced in artificial culture media and for that present in urine which has undergone ammoniacal fermentation (§ 484). As urea is vigorously attacked by alkalies, the material is distilled with magnesia, which is without action on urea at temperatures below 60°; and, even at 100°, has but a very feeble action, which is proportional to the length of time the heating is continued (Berthelot and André).

534. In the distilling-flask of Aubin's apparatus (§ 39) are placed 5 cc. of urine; to this are added 150-200 cubic

centimetres of distilled water, and a few grammes of freshly calcined magnesia in fine powder. The mixture is distilled and the distillate titrated with N/5 sulphuric acid, using methyl orange as indicator.

To allow for the small quantity of ammonia resulting from the action of magnesia upon urea the operation is stopped when the amount of ammonia distilling over is very small and is proportional to the time of heating. The time during which the urea-containing liquid has been boiled is noted (about  $\frac{3}{4}$  hour).

Next, a volume of distilled water equal to that of the distillate which has passed over is added to the contents of the distilling-flask, and the distillation repeated for exactly the same length of time as the first operation. The quantity of ammonia liberated and determined by titration as before corresponds to that set free from urea in the first distillation. By deducting this figure from that first obtained we have the amount of ammonia originally present, as distinct from urea.

535. If absolutely accurate results are required the distillation with magnesia must be carried out *in vacuo* at a temperature below 50°. A flask containing a known excess of standard sulphuric acid is fitted to the Aubin apparatus, and by titrating this after the distillation is completed the amount of ammonia which has passed over can be determined.

536. *Estimation of Ammonia and Amino-Acids in Urine.*—When a rapid and approximate estimation of ammonia in urine is required the following method may be used, and is sufficiently accurate for clinical purposes (Ronchèse). It must be borne in mind, however, that amino-acids are estimated along with ammonia, and these may not form an entirely negligible factor in certain pathological conditions.

537. To 10 cubic centimetres of urine are added 100 cubic centimetres of boiled water and a few drops of phenolphthalein. Sufficient decinormal soda is added from a pipette to produce a very faint pink colour. Then 20 cubic centimetres of commercial formalin (40 per cent

formaldehyde) are diluted with an equal volume of water exactly neutralized, and added to the diluted urine. Decinormal soda is then run in to the mixture until a faint pink colour is observed.

In this reaction formaldehyde reacts with ammonium salts to form hexamethylenetetramine, and the acids originally in combination with the ammonia are set at liberty.

The volume  $n$  of decinormal soda which is used corresponds then to the amount of ammonia originally present. In consequence of the inhibitory action exercised upon the colour-change by ammonium salts the figure obtained must be increased by  $1/30$  (Ronchèse). The quantity of ammonia per litre of urine is then  $n \times 31/30 \times 0.017 \times 100 = n \times 1.757$ .

### Detection of Indole

538. Certain micro-organisms, notably some putrefactive bacteria, the colon bacillus, and the cholera vibrio, etc., can produce indole in considerable amounts.

The presence of this body may be suspected when cultures give off the characteristic smell of indole (odour of dental caries); its presence may be established by the following reactions:

539. To 10 cubic centimetres of culture fluid add 1 cc. of a 1 in 10,000 solution of sodium nitrate, and then very carefully, and with gentle shaking, 20-25 drops of concentrated sulphuric acid. A pink or red colour—due to nitroso-indole—is produced.

If the tint is masked by the colour of the original fluid, 2 cc. of amyl alcohol are added and the mixture shaken. Amyl alcohol dissolves the red colouring matter and forms a red supernatant layer.

Should the culture medium itself contain traces of nitrates these may be reduced to nitrites at the same time as indole is formed. In such cases the characteristic red colour is given on the addition of sulphuric acid alone. The culture of the cholera vibrio (in "peptone water")

affords a well-known example of this, whence the phenomenon is known as the "cholera red reaction."

540. When only traces of indole are present, it may be separated by taking advantage of the fact that it is volatile in a current of steam. The apparatus described in § 183 may be used. The liquid to be tested is rendered slightly acid with a little hydrochloric acid and put into the distilling-flask of the apparatus (D. Fig. 24), and the distillation carried out in the manner previously described. The distillate may contain skatole, phenols, fatty acids and other substances as well as indole. It is now rendered slightly alkaline with soda, replaced in the distilling-flask, and the distillation repeated. Indole may be recognized in the distillate by means of *p*-dimethylaminobenzaldehyde (P. Ehrlich). To 10 cubic centimetres of distillate are added 8-10 drops of a .5 per cent alcoholic solution of this reagent, then drop by drop, concentrated sulphuric acid (about 1 cc. in all). A fine rose-pink colour develops. The acid may also be added so as to form a layer at the bottom of the tube; a violet-red ring then forms at the line of demarcation of the two liquids.

541. Indole, when treated with sodium  $\beta$ -naphthoquinonemonosulphonate in an alkaline medium, gives a deep blue coloration (Hertes). To 10 cubic centimetres of a very dilute solution of indole are added one drop of 10 per cent potash solution and 1 or 2 drops of a 2 per cent watery solution of the  $\beta$ -naphthoquinonemonosulphonate. A blue colour gradually develops, the reaction being assisted by heating. On shaking the mixture with 1 or 2 cc. of chloroform this assumes a red colour, while the watery layer is colourless.

If alcohol is present in any quantity the tint obtained will be greenish and not blue.

### Detection of Skatole

542. Skatole often accompanies indole in putrefactive processes; it has a disgusting faecal odour, and like indole

is volatile in a current of steam. It does not give any colour reaction with sulphuric acid and sodium nitrite.

543. Treated with *p*-dimethylaminobenzaldehyde and sulphuric acid as in § 540, skatole gives a violet coloration which after a time becomes blue.

544. If to a few cubic centimetres of a solution containing skatole there is added one drop of a 10 per cent solution of potash, and then one or two drops of sodium  $\beta$ -naphthoquinonemonosulphonate, the liquid becomes orange, the tint deepening on heating. This colouring matter is not extracted by chloroform.

## CHAPTER XXIII

### SYNTHETIC PHENOMENA

#### Assimilation of Elements

##### Cultivation of “*Aspergillus niger*” in Raulin’s Solution

545. RAULIN’S solution, as a culture medium, possesses the distinction of providing, in a simple mineral form—except as regards saccharose and tartaric acid—all the elements necessary for the nutrition of *Aspergillus niger*. The saccharose supplies the needed carbon and hydrogen, as also by its combustion the energy necessary for the growth of the plant; the tartaric acid, the faint acidity which is favourable to the growth of such moulds as *Aspergillus*. The influence of any particular element upon growth can be studied by omitting it from the mixture and observing the development of the plant under these conditions. The effects, for instance, of phosphoric acid and potassium can be readily studied.

546. For this purpose the following nine solutions are prepared :

I.	{ Tartaric acid . . . . .	2·667 per cent
	{ Magnesium carbonate . . . . .	0·267 „
2.	Ammonium nitrate . . . . .	2·667 „
3.	Ammonium phosphate . . . . .	0·4 „
4.	Potassium carbonate . . . . .	0·4 „
5.	Ammonium sulphate . . . . .	0·167 „
6.	Zinc sulphate . . . . .	0·0467 „
7.	Ferrous sulphate . . . . .	0·0467 „
8.	Sodium silicate . . . . .	0·0467 „

and lastly, a solution containing 35 grammes of saccharose in 150 cc. of distilled water.

To prepare Raulin's solution in its entirety 50 cc. of the sugar solution are taken in a beaker, and 25 cc. of each of the other eight solutions are added, the whole being well stirred. Thus 250 cc. of the mixture are obtained, which are turned into a photographic developing dish 13 cm. + 18 cm.

To prepare the solution without potassium, the 25 cc.

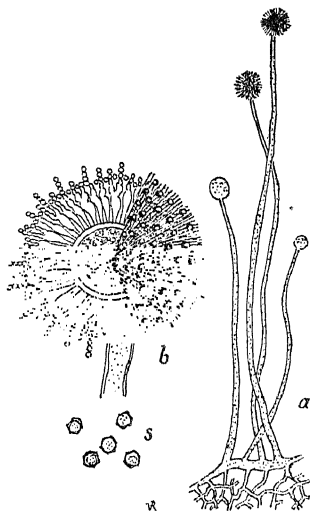


FIG. 59.—*Aspergillus Niger*.

(a) Conidiophores.

(b) Enlarged head of Conidiophore.

(c) Spores.

of potassium carbonate solution are replaced by 25 cc. of either distilled water or .3 per cent sodium carbonate solution. Similarly, to prepare a solution without phosphoric acid, the 25 cc. of ammonium phosphate are replaced by an equal volume of either distilled water or of .4 per cent ammonium sulphate.

547. The three solutions being thus prepared and transferred to appropriate dishes, each receives 10 drops of a suspension of *Aspergillus* spores in a little water. The contents of each dish are stirred so as to secure a fairly uniform



distribution of the spores, and the dishes are put in the 37° incubator. To prevent undue evaporation, while at the same time permitting free access of air, each dish may be covered with a piece of glass supported on a glass rod bent into a V shape.

At the end of 24 hours, commencing growths of white mycelium are seen. After three or four days, growth has entirely ceased and the surface of the solution containing both potassium and phosphoric acid is covered with a thick resistant layer of growth; the surfaces of the two remaining solutions on the other hand show only isolated patches of growth, and these are small and exhibit but little coherence. These various mycelia can be removed, drained of liquid, and dried in the air oven till constant weight is obtained. The relations between the various weights will give an idea of the importance to growth of the element wanting in the incomplete solution. Microscopically the mould presents the appearances shown in Fig. 59.

### Assimilation by Chlorophyll

#### Liberation of Oxygen

548. Green plants, owing to the presence of chlorophyll, can decompose carbon-dioxide under the action of light. In the presence of water there is a fixation of carbon with liberation of oxygen and a transitory production of formaldehyde.

This liberation of oxygen is easily demonstrable. In a large tube of about half a litre capacity, closed at one end, are placed some branches with their attached leaves of water plants, such as *Potamogeton* or *Elodeum*. The vessel is filled with water nearly saturated with carbon-dioxide (Seltzer water diluted with four or five times its volume of water), and inverted in a vessel filled with the same solution. If the whole is exposed to the action of direct sunlight, the leaves of the contained plants are seen to be covered with small bubbles of gas, which are gradually

detached and collect in the uppermost part of the tube. The gas so collected is oxygen with a small admixture of carbon-dioxide.

When a sufficiency has been obtained, the gas is collected in a test-tube with the assistance of a funnel and pneumatic trough. A potash pastille is introduced into the test-tube, which is then closed with the thumb and shaken, so as to cause absorption of the carbon-dioxide. If the test-tube is now held mouth upwards and a glowing splinter of wood is introduced, it immediately bursts into flame, thus indicating the presence of oxygen.

### Production of Volatile Aldehydes in Green Leaves

549. The formic aldehyde which is formed from the fixation of carbon by green leaves is accompanied by ordinary (acetic) aldehyde, and by other volatile aldehydes of greater complexity. The formation of these bodies is easily demonstrated by distilling the leaves with water. In a flask of about 1 litre capacity are placed *fresh* leaves of beech, chestnut, or lime, etc., care being taken that they are not packed too tightly. Sufficient water is added to two-thirds fill the flask, which is then connected to the Aubin apparatus and the contents distilled. The distillate will be found to reduce ammoniacal silver nitrate solution and Nessler's reagent, as occurs with the majority of aldehydes.

550. If to 5 cc. of the distillate is added an equal volume of aniline water (prepared by shaking a few drops of aniline with distilled water and filtering through a moistened filter), after a few minutes a milky appearance is developed due to the precipitation of condensation products of aniline with the aldehydes.

### Reactions of Formic Aldehyde

551. Formic aldehyde causes energetic reduction of ammoniacal silver nitrate and of Nessler's reagent. With a solution of aniline it gives a milk-white turbidity. These reactions are common to the other aldehydes of the series.

552. To 5 cc. of the solution to be tested for formic aldehyde add 2 cc. of a fresh filtered 1 per cent solution of phenylhydrazine hydrochloride, 1 cc. of fresh 5 per cent potassium ferricyanide, and 5 cc. of pure hydrochloric acid. If formic aldehyde is present a fine fuchsia-red coloration is produced.

553. The above reaction is produced, even in the presence of various pigments, if the following technique is adopted. If, for instance, the test is applied to an alcoholic extract of green leaves containing a good deal of chlorophyll, the solution is first gently heated in the water-bath to evaporate off the alcohol. The residue is taken up with 10 cc. of water, and this extract is tested as in § 552. The liquid so obtained is diluted with twice its volume of water and shaken up with 5 to 10 cc. of ether. The red salt undergoes dissociation, and the base is taken up by the ether, which has then a pale yellow tint and is decanted into a test-tube. To it are added 1 to 2 cc. of concentrated hydrochloric acid, when a fine red colour is produced owing to the formation of the hydrochloride (Schryver).

554. Formaldehyde forms a condensation product with dimethylaniline, giving a body of the formula  $\text{CH}_2 : (\text{C}_6\text{H}_4\text{N} \begin{smallmatrix} \text{CH}_3 \\ \text{CH}_3 \end{smallmatrix})_2$ , which on oxidation gives a blue colour (A. Trillat).

To carry out the test the following procedure is adopted. The liquid containing the aldehyde is distilled, and 50 cc. of the distillate are taken. These are introduced into a stoppered flask of about 75 cc. capacity with 1 cc. of pure dimethylaniline (recently distilled, B.P. 191-192°), and 10 to 15 drops of 10 per cent sulphuric acid. The flask is stoppered and kept at 75-80° on the water-bath for half an hour with occasional shaking.

The condensation being finished, the liquid is rendered definitely alkaline with soda solution, and any excess<sup>1</sup> of dimethylaniline removed by a current of steam; this

<sup>1</sup> If any excess of dimethylaniline remains, an evanescent green colour is first obtained instead of blue in the subsequent test.

requires about 5-10 minutes. The turbid liquid is then filtered, and the slight insoluble residue suspended in 5-10 cc. of water acidulated with acetic acid. A few cubic centimetres of a suspension of lead dioxide (2 grammes to 100 cc.  $H_2O$ ) are next added, and the whole brought to boiling. In the presence of formic aldehyde the liquid assumes a blue colour.

555. The above reaction can easily be applied to the detection of methyl alcohol. In this case the liquid under investigation is first oxidized, 50 cc. being mixed with 10 cc. of 50 per cent sulphuric acid with the addition of 5 grammes of powdered potassium bichromate. After solution of this latter the liquid is distilled and the distillate tested for formic aldehyde. If a specimen of ordinary alcohol is under examination the first few cubic centimetres of distillate are rejected, as they contain mainly ordinary (acetic) aldehyde.

### Fixation of Nitrogen by Bacteria

556. The free nitrogen of the air is fixed by several species of bacteria by which it is used for the synthesis of protein substances. This occurs in the case of *Bacillus radicola*, which occurs in the little nodules on the roots of the pea and other plants belonging to the natural order *Leguminosae*. This fixation may be demonstrated by cultures of the organism on media containing sugar.

557. *Preparation of Culture Medium*.—A suitable medium is prepared by boiling 50 grammes of white haricots with 250 cc. of water in a flask until they commence to burst. The liquid is filtered through a strainer and made up to 250 cc., 5 grammes of saccharose are dissolved in it and the mixture is filtered. Exactly 100 cc. of this haricot bouillon are poured into a flat-bottomed flask of one litre capacity so as to form a layer of about 1-2 cm. in depth. A second flask is similarly prepared, and both are sterilized in the autoclave at  $110^\circ$  for 20 minutes.

558. *Isolation of Bacteria from the Nodules*.—The remaining 50 cc. of haricot bouillon are turned into a flask and

·75 grammes of agar (cut into fragments with scissors) are added. If necessary the mixture is carefully neutralized with a few drops of dilute soda, boiled gently for half an hour, and then kept at  $120^{\circ}$  in the autoclave for a further half-hour. The liquid is filtered boiling through Chardin's paper and distributed into test-tubes (4-5 cc. in each tube), which are then sterilized at  $110^{\circ}$  for 20 minutes and allowed to cool in an inclined position so as to form "slopes."

To isolate the organism the root parts of pea or vetch showing nodules are taken. These are washed and dried, and the outside of each nodule is gently burnt with a

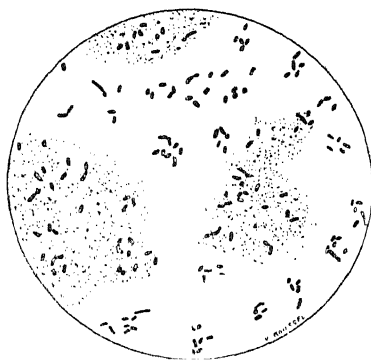


FIG. 60.—Bacteria from Nodules on Roots of *Leguminosae*.

stirring rod heated to redness so as to sterilize it. The point of a pipette is thrust through the burnt part so as to reach the centre of the nodule; a drop of fluid is then drawn up in the pipette, and the surfaces of several slopes are inoculated (§ 462). This liquid, smeared on a slide, dried, fixed and stained with dilute fuchsin, shows quantities of the bacilli of the nodules (Fig. 60).

The inoculated tubes are kept at a temperature which should not exceed  $25^{\circ}$ . After four or five days, transparent viscous-looking colonies make their appearance; these slowly increase in size and gradually cover the surface of the medium, forming a thick layer.

559. *Cultivation, and Estimation of Fixed Nitrogen.*—One

of the two flasks (§ 557) is inoculated from a colony of *B. radiculicola* with the usual precautions: the other one serves as a control. The two flasks are kept at about 25° for at least a month.

At the end of this time it will be seen that the organism has developed, forming an abundant viscous mass which fills the liquid. It has used the sugar in the course of its development, and at the same time has "fixed" nitrogen from the air.

The amount of nitrogen absorbed may be estimated by emptying the contents of both flasks into an evaporating basin, washing the flasks out and adding each washing to its appropriate basin, and then evaporating to about 10 cc. on the water-bath. The liquid is turned into a flask of about 125 cc. capacity, the basin washed out with 5 cc. of sulphuric acid, which is then added to the contents of the flask; the washing with acid is then repeated twice again. Finally, a drop of mercury is added and the nitrogen estimated as shown in § 38.

The difference between the quantities of nitrogen found in the culture and in the control gives the amount of nitrogen fixed.

# TABLES

## ATOMIC WEIGHTS OF THE CHIEF ELEMENTS

Element.	Symbol.	Atomic Weight.	Element.	Symbol.	Atomic Weight.
Aluminium .	Al	27.1	Molybdenum .	Mo	96.0
Antimony .	Sb	120.2	Neodymium .	Nd	144.3
Argon . .	Ar	39.88	Neon . .	Ne	20.2
Arsenic . .	As	74.96	Nickel . .	Ni	58.68
Barium . .	Ba	137.37	Niton . .	Nt	222.4
Bismuth . .	Bi	208.0	Nitrogen .	N	14.01
Boron . .	Bo	11.0	Osmium . .	Os	190.9
Bromine . .	Br	79.92	Oxygen . .	O	16.0
Cadmium . .	Cd	112.40	Palladium .	Pd	106.7
Cæsium . .	Cs	132.81	Phosphorus .	P	31.10
Calcium . .	Ca	40.07	Platinum . .	Pt	195.2
Carbon . .	C	12.0	Potassium .	K	39.1
Cerium . .	Ce	140.25	Præseodymium	Pr	140.6
Chlorine . .	Cl	35.46	Radium . .	Ra	226.4
Chromium .	Cr	52.0	Rhodium . .	Rh	102.9
Cobalt . .	Co	58.97	Rubidium . .	Rb	85.45
Columbium .	Cb	93.5	Ruthenium .	Ru	101.7
Copper . .	Cu	63.57	Samarium . .	Sa	150.4
Dysprosium .	Dy	162.5	Scandium . .	Sc	44.1
Erbium . .	Er	167.7	Selenium . .	Se	79.2
Europium . .	Eu	152.0	Silicon . .	Si	28.3
Fluorine . .	F	19.0	Silver . .	Ag	107.88
Gadolinium .	Gd	157.3	Sodium . .	Na	23.0
Gallium . .	Ga	69.9	Strontium . .	Sr	87.63
Germanium .	Ge	72.5	Sulphur . .	S	32.07
Glucinum . .	Gl	9.1	Tantalum . .	Ta	181.5
Gold . .	Au	197.2	Tellurium . .	Te	127.5
Helium . .	He	3.99	Terbium . .	Tb	159.2
Hydrogen . .	H	1.008	Thallium . .	Tl	204.2
Indium . .	In	114.8	Thorium . .	Th	232.4
Iodine . .	I	126.92	Thulium . .	Tm	168.5
Iridium . .	Ir	193.1	Tin . .	Sn	119.0
Iron . .	Fe	55.84	Titanium . .	Ti	48.1
Krypton . .	Kr	82.92	Tungsten . .	W	184.0
Lanthanum .	La	139.0	Uranium . .	U	238.5
Lead . .	Pb	207.1	Vanadium . .	V	51.0
Lithium . .	Li	6.94	Xenon . .	Xe	130.2
Lutecium . .	Lu	174.0	Ytterbium . .	Yb	172.0
Magnesium .	Mg	24.32	Yttrium . .	Yt	89.0
Manganese .	Mn	54.93	Zinc . .	Zn	65.37
Mercury . .	Hg	200.6	Zirconium . .	Zr	90.6

## TABLES

## SPECIFIC GRAVITIES CORRESPONDING TO BEAUMÉ DEGREES

*Liquids heavier than Water*

Beaumé Degree.	Specific Gravity.	Beaumé Degree.	Specific Gravity.	Beaumé Degree.	Specific Gravity
0	1.000	23	1.190	46	1.468
1	1.007	24	1.200	47	1.483
2	1.014	25	1.210	48	1.498
3	1.022	26	1.220	49	1.514
4	1.029	27	1.231	50	1.530
5	1.037	28	1.241	51	1.546
6	1.045	29	1.252	52	1.563
7	1.052	30	1.263	53	1.580
8	1.060	31	1.274	54	1.597
9	1.067	32	1.285	55	1.615
10	1.075	33	1.297	56	1.634
11	1.083	34	1.308	57	1.652
12	1.091	35	1.320	58	1.671
13	1.100	36	1.332	59	1.691
14	1.108	37	1.345	60	1.710
15	1.116	38	1.357	61	1.732
16	1.125	39	1.370	62	1.753
17	1.134	40	1.383	63	1.775
18	1.142	41	1.397	64	1.796
19	1.152	42	1.410	65	1.820
20	1.162	43	1.424	66	1.842
21	1.171	44	1.438	67	1.865
22	1.180	45	1.453	..	..

*Liquids lighter than Water*

Beaumé Degree.	Specific Gravity.	Beaumé Degree.	Specific Gravity.	Beaumé Degree.	Specific Gravity.
10	1.000	21	0.927	40	0.823
11	0.993	22	0.921	45	0.800
12	0.986	23	0.915	50	0.778
13	0.979	24	0.909	55	0.757
14	0.972	25	0.903	60	0.737
15	0.965	26	0.897	70	0.700
16	0.959	27	0.892	80	0.666
17	0.952	28	0.886	90	0.636
18	0.946	29	0.880	100	0.609
19	0.939	30	0.875	..	..
20	0.933	35	0.848	..	..



SPECIFIC GRAVITIES OF AQUEOUS SOLUTIONS OF AMMONIA  
AT 15° (Lunge and Viernik)

Specific Gravity.	Grammes of $\text{NH}_3$ .	
	In 100 gr.	In 100 cc.
0.990	2.31	2.29
0.980	4.80	4.70
0.970	7.31	7.09
0.960	9.91	9.51
0.950	12.74	12.10
0.940	15.63	14.69
0.930	18.61	17.34
0.926	19.87	18.42
0.924	20.49	18.93
0.922	21.12	19.47
0.918	22.39	20.56
0.914	23.68	21.63
0.910	24.99	22.74
0.906	26.31	23.83
0.902	27.65	24.94
0.898	29.01	26.05
0.894	30.37	27.15
0.890	31.75	28.26
0.886	33.25	29.46
0.882	34.95	30.83

SPECIFIC GRAVITIES OF AQUEOUS SOLUTIONS OF POTASH  
AT 15° (Lunge)

Specific Gravity.	Beaumé Degree.	Grammes of KOH.	
		In 100 gr.	In 100 cc.
1.014	2	1.7	1.7
1.029	4	3.5	3.6
1.045	6	5.6	5.8
1.060	8	7.4	7.8
1.075	10	9.2	9.9
1.091	12	10.9	11.9
1.108	14	12.9	14.3
1.125	16	14.8	16.7
1.142	18	16.5	18.8

SPECIFIC GRAVITIES OF AQUEOUS SOLUTIONS OF POTASH  
AT 15° (Lunge)—*continued*

Specific Gravity.	Beaumé Degree.	Grammes of KOH.	
		In 100 gr.	In 100 cc.
1·162	20	18·6	21·6
1·180	22	20·5	24·2
1·200	24	22·4	26·9
1·220	26	24·2	29·5
1·241	28	26·1	32·4
1·263	30	28·0	35·3
1·285	32	29·8	38·5
1·308	34	31·8	41·6
1·320	35	32·7	43·2
1·332	36	33·7	44·9
1·345	37	34·9	46·9
1·357	38	35·9	48·7
1·383	40	37·8	52·2
1·410	42	39·9	56·3
1·438	44	42·1	60·5
1·468	46	44·6	65·5
1·498	48	47·1	70·6
1·530	50	49·4	75·6
1·563	52	51·9	81·1
1·597	54	54·5	87·0
1·634	56	57·5	94·0

SPECIFIC GRAVITIES OF AQUEOUS SOLUTIONS OF SODA  
AT 15° (Lunge)

Specific Gravity.	Beaumé Degree.	Grammes of NaOH.	
		In 100 gr.	In 100 cc.
1·014	2	1·20	1·20
1·029	4	2·71	2·80
1·045	6	4·00	4·20
1·060	8	5·29	5·60
1·075	10	6·55	7·00
1·091	12	8·00	8·70
1·108	14	9·42	10·40
1·125	16	10·97	12·30
1·142	18	12·64	14·40
1·162	20	14·37	16·70
1·180	22	15·91	18·80
1·200	24	17·67	21·20

PRESSURE OF AQUEOUS VAPOUR IN MILLIMETRES OF MERCURY  
BETWEEN 10° AND 30°

Temperature.	Pressure.	Temperature.	Pressure.
10°	9·1	21°	18·5
11°	9·8	22°	19·6
12°	10·4	23°	20·8
13°	11·1	24°	22·1
14°	11·9	25°	23·5
15°	12·7	26°	25·0
16°	13·5	27°	26·5
17°	14·4	28°	28·1
18°	15·3	29°	29·7
19°	16·3	30°	31·5
20°	17·4	..	..

SOLUBILITIES OF SOME COMMON SUBSTANCES AT DIFFERENT  
TEMPERATURES IN 100 PARTS OF WATER

	0°.	20°.	40°.	60°.	100°.
Ba(OH) <sub>2</sub> + 8H <sub>2</sub> O . . .	..	7·4	16·5	48	..
Na <sub>2</sub> CO <sub>3</sub> + 10H <sub>2</sub> O . . .	21	93	1142 (at 38°)	..	540
NaHCO <sub>3</sub> . . . . .	9	11	13	15·6	..
BaCl <sub>2</sub> . . . . .	31	36	41	46	59
HgCl <sub>2</sub> . . . . .	5·7	7·4	9·6	13·7	54
NaCl . . . . .	35·5	36	36·6	37·2	39·6
NH <sub>4</sub> Cl . . . . .	28	37	46	55	73
AgNO <sub>3</sub> . . . . .	122	230	390	540	940
KNO <sub>3</sub> . . . . .	13	31	64	111	247
Na <sub>2</sub> HPO <sub>4</sub> + 12H <sub>2</sub> O . . .	..	17·2	..	..	>300
CaSO <sub>4</sub> + 2H <sub>2</sub> O . . . . .	0·19	0·20	0·21	0·21	0·17
CuSO <sub>4</sub> + 5H <sub>2</sub> O . . . . .	31·6	42·3	56·9	77·3	203·3
MgSO <sub>4</sub> + 7H <sub>2</sub> O . . . . .	76·9	119·8	179·5	..	671·2
Na <sub>2</sub> SO <sub>4</sub> + 10H <sub>2</sub> O . . . .	12·2	58·3	412 (at 34°)	..	..
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> . . . . .	71·0	76·3	81·6	86·9	97·5
ZnSO <sub>4</sub> + 7H <sub>2</sub> O . . . . .	115	161	224	313	654
Potassium Bitartrate . .	0·3	0·6	1·3	2·4	6·9
Oxalic Acid . . . . .	5·2	13·9	35	75	>350

SOME USEFUL CONSTANTS RELATING TO SUGARS AND  
THEIR DERIVATIVES

	Melting Point.	$[\alpha]_D$ in 10 % solutions.
<i>l</i> -xylose . . . .	165-166°	+18.84
<i>l</i> -arabinose . . . .	159-160°	+102.65
Rhamnose . . . .	108°	+9.1
<i>d</i> -fructose (or <i>l</i> ævulose) .	95° (about)	-101.38 + 0.56 <i>t</i> <sup>1</sup>
<i>d</i> -glucose . . . .	144-146°	+52.5
<i>d</i> -galactose . . . .	162°	+84.36 - 0.21 <i>t</i>
<i>d</i> -mannose . . . .	132°	+13.92
<i>d</i> -sorbose . . . .	178-179°	-42.80
Saccharose . . . .	160° (about)	+66.50
Lactose . . . .	204°	+54.52
Maltose . . . .	..	+130.5
Mannose-hydrazone . . .	198-199°	
Xylosazone . . . .	166°	
Arabinosazone . . . .	143°	
Glucosazone . . . .	230-232°	
Galactosazone . . . .	214°	
Sorbosazone . . . .	159°	
Lactosazone . . . .	200° (about)	
Maltosazone . . . .	206°	
Mannitol . . . .	168°	
Inosite (inactive) . . .	224°	

CONSTANTS OF SOME COMMON FATTY BODIES

	Index of Saponification.	Iodine Index.	Specific Gravity at 15°.
Triacetin . . . .	772.0	..	1.155
Tributylin . . . .	557.3	..	1.052
Monobutylin . . . .	346.3	..	1.088
Linseed oil. . . .	192-195	173-201	0.932-0.937
Nut oil . . . .	195	145	0.925-0.926
Poppy seed oil . . . .	195	133-143	0.924-0.927
Cotton seed oil . . . .	193-195	108-110	0.922-0.930
Earth nut oil . . . .	190-196	83-100	0.916-0.920
Sweet almond oil . . .	191	96-99	0.918-0.920

<sup>1</sup> In these formulæ *t* indicates the temperature expressed in degrees centigrade. The rotatory powers are calculated by *algebraical sum* of the invariable term and the product of the other term with the temperature.

CONSTANTS OF SOME COMMON FATTY BODIES—*continued*

	Index of Saponification.	Iodine Index.	Specific Gravity at 15°.
Olive oil . . .	185-196	79-88	0.916-0.920
Castor oil . . .	183-186	83-86	0.963-0.968
Oil of maize . . .	188-193	111-130	0.920-0.925
Colza oil . . .	170-179	94-102	0.914-0.917
Cod liver oil . . .	171-189	167	0.922-0.927
Neats foot oil . . .	194.3	69.3-70.4	0.913-0.916
Palm oil . . .	196-202	51.5	0.921-0.925
Cocoa-oil (coprah) . .	246-260	8-9.5	0.925-0.926
Cocoa butter . . .	193.5	32-41	0.964-0.976
Butter . . .	227	26-38	0.910-0.913
Beef suet . . .	193-200	38-46	0.943-0.952
Lard . . .	195.4	50-70	0.934-0.938
Beeswax . . .	90-98	7.9-11	0.962-0.966



## TRANSLATOR'S NOTES

1. *Maquenne's Block* (p. 53).—The block consists of a mass of copper, or of an alloy of copper and zinc ("laiton"); this is traversed by a horizontal tunnel situated just beneath the surface, serving for the reception of the thermometer and permitting of the entire mercury column of the latter being contained in the block of metal. The apparatus is heated from below by a row of gas-burners connected with a fine adjustment tap, which allows of a fixed temperature being maintained. The upper surface of the block is polished and has a number of little cavities, into which samples of the materials under investigation may be placed.

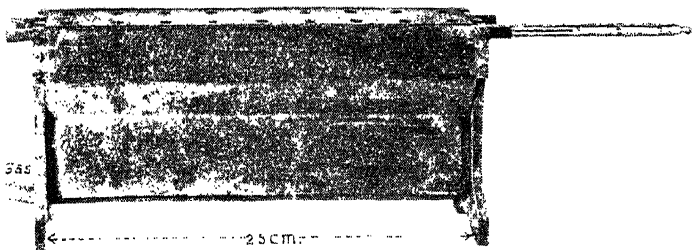


FIG. 61.—Maquenne's Block.

2. *Classification of Proteins* (p. 156).—Various schemes of classification of these bodies have been proposed. In English the term proteid is often used loosely as synonymous with protein; it will be noticed that here MM. Bertrand and Thomas make use of it to define a special group of protein bodies.

3. *Precipitation of Proteins by Metallic Salts* (p. 157).—The composition of these precipitates varies under different conditions of admixture of the same salt and the same protein, and accordingly the results of the analysis of such precipitates are not very concordant. If, for example, a 3 per cent albumin solution is added drop by drop to a decinormal cupric sulphate solution, a dense precipitate results; that, however, redissolves on the addition of excess of the protein solution. On again adding the copper sulphate

solution a further precipitate occurs, which in turn dissolves on the addition of more protein.

If, on the other hand, the albumin solution is run into a *concentrated* solution of cupric sulphate, a precipitate forms, which dissolves on shaking. On further addition of protein this precipitate dissolves with increasing difficulty, until eventually a permanent precipitate is produced. (See Schryver, *The General Characters of the Proteins*. Longmans. London.)

4. *On the Use of Ammonia in performing the Biuret Reaction* (p. 159).—It is generally stated that the addition of copper sulphate and ammonia to solutions of proteins gives rise to a violet colour. This is probably because the amount of copper used is too large; and if very dilute copper solution is used, some peptones and albumoses give a very fine pink. Using 1 cc. of 1 per cent protein solution, 1 cc. of N/100 copper sulphate solution, and 10 cc. of ammonium hydrate solution, it was found that egg-white and gelatine produced no effect, beyond the fact that their addition caused a diminution in the depth of the faint blue colour resulting from the admixture of the copper and ammonia; and indeed, if sufficient protein solution is added, this colour may be made to disappear altogether.

Using a 1 per cent solution of Witte peptone the result is different, and a definite pink tint results; this is practically identical with that obtained when potash or soda is employed.

On attempting to see what constituents of the Witte peptone (which consists mainly of a mixture of albumoses) gave this reaction, some interesting results were obtained. To a 2 per cent solution of Witte peptone was added an equal volume of absolute alcohol; the mixture was allowed to stand for twenty-four hours, and the precipitate separated by filtration. The filtrate contains  $\beta$ -proto- and  $\beta$  deuterio-proteose. The precipitate is collected and mixed with water; a portion redissolves, this consists of  $\alpha$  proto- and  $\alpha$  deuterio-proteose; the insoluble residue consists of crude hetero-proteose. When these three fractions were examined by the copper-ammonia method the hetero-proteose fraction alone failed to give a pink colour, the proto- and deuterio-proteose mixtures both giving a fine pink.

It seemed to me that this method might be made use of in examining suitable test-meals, to test the efficiency of gastric digestion. If milk be treated with dilute hydrochloric acid placed in the 37° incubator for several hours, removed and filtered, the filtrate will give no colour reaction when added to such a copper-ammonia mixture as described. If on the other hand milk be subjected to gastric digestion and then examined at above (*e.g.* in a vomit), the pink colour indicating the presence of digestion products is obtained. The biuret reaction has been quantitatively used by H. M. Vernon, and possibly by selecting suitable standards some quantitative determinations upon the amount of gastric digestion might be



obtained. In one case of a specimen of a vomited milk-meal, in which free HCl could not be found, the filtered matter failed to give the ammonia-copper test for proteoses and peptones.

(Colwell. "Notes on the Biuret Reaction for Proteins," *Arch. Middlesex Hosp.*, p. xxiii., 1911.)

5. *Hydrogen-ion Concentration*.—A simple account of hydrogen-ion concentration, with references to literature, is contained in a pamphlet on "The Reaction of Media," published by the Medical Research Committee. Price 6d. Special Report Series No. 35.



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